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Role of *traM* in F Conjugation

by

Jun Lu ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Microbiology and Biotechnology

Department of Biological Sciences

Edmonton, Alberta

Fall 2004

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Acknowledgements

First of all, I would like to thank my supervisor Dr. Laura Frost for her guidance and support. She not only provided me with insightful advice, crucial in fulfilling this research project, but also took great pains to help me improve my English writing and presentation skills. Her great personality and knowledge inspired me and made my study in Canada both enjoyable and productive.

My supervisory committee members, Drs. Diane Taylor and William Page, also gave me great help throughout my Ph.D. program and contributed to the revision of this thesis. I would also like to thank Drs. Mark Glover, Brenda Leskiw, and Richard Meyer for participating in my Ph.D. thesis defense.

Members of the Frost lab have always supported me and made our lab fun. Dr. Richard Fekete helped me start the TraM project and contributed to Chapter 4 of this thesis. Jan Manchak provided excellent technical support and also contributed to Chapter 8 of this thesis together with Colin Davidson and Dr. William Klimke. Ryan Will shared many interesting ideas with me, and his great sense of humor made the lab full of laughter. Dr. Mike Gubbins, Isabella Lau, Trevor Elton, Candace Rypien, Dr. Perrin Beatty, Barbara Klinger, Dr. Samantha Holland, and many project and summer students throughout the years have all been a great pleasure to work with.

Many other colleagues have helped me in different aspects over the years. Dr. Frank Nargang and Suzanne Hoppins assisted me in blue native gel analysis of TraM mutants. Troy Locke and Jody Butt taught me FPLC techniques. Drs. Neville Firth and Ronald Skurray contributed the sequence of pED208 *traG* and revised Chapter 8 of this

thesis. Our neighbors, members of Leskiw lab, provided me with various help during my daily lab work.

I am grateful to the Department of Biological Sciences for accepting me and providing an excellent Ph.D. program and research environment. I would also like to thank the Alberta Ingenuity Fund for supporting me with a studentship as well as the National Science and Engineering Research Council and the Canadian Institutes for Health Research for funding our lab.

My family and friends gave me great support during my study in Canada. My parents Lu Yuansun and Qi Cuiyun have always believed in me and have been unconditionally supportive of my career choices. Otherwise, I don't think I would have come this far. My younger brothers Lu Ting and Lu Ye have been taking care of my parents in China, which makes my life here easier. I am grateful to my parents-in-law Zhao Zhenggang and Li Hui, and my brother-in-law Zhao Hui for supporting my wife and I moving to Canada. I would also like to thank my long-time friend Dr. Haojie Huang for continuous encouragement.

Finally, I want to dedicate this thesis to my beautiful wife, Wen Zhao. She has been taking very good care of me here and has put up with my long hours in the lab. Also, Wen volunteered to help me with my research during many weekends and did most of the sequencing and donor ability assays in Chapters 4 of this thesis.

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Abbreviations

A	Adenine
A_{420}	Absorbance at 420 nm
Aa	Amino acids
Amp	Ampicillin
ATP	Adenosine triphosphate
BCIG	5-brome-4-chloro-3-indolyl-β-D-galactoside
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BSA	Bovine serum albumin
CA	Carbonic anhydrase
CAP	Catabolite activator protein
CEA	Chicken egg albumin
CFU	Colony forming units
Cm	Chloramphenicol
Cyto. C	Cytochrome C
Da	Daltons
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
ds	Double strand
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assays
F	Fertility factor
FPLC	Fast Performance Liquid Chromatography
g	Gravity
g	Grams
G	Guanine

IHF	Integration host factor
HSF	Helicase superfamily
H-NS	Heat-stable nucleoid structuring protein
HU	Heat-unstable nucleoid protein
IPTG	Isopropyl β-D-thiogalactopyranoside
kb	Kilobase pairs
kDa	Kilodaltons
Km	Kanamycin
LB	Luria-Bertani
M	Molars
mA	Milliamperes
mg	Milligrams
ml	Milliliters
mM	Millimolars
Mpf	Mating pair formation
MU	Miller Units
Nal	Nalidixic acid
NCBI	National Center for Biotechnology Information
Ni-NTA	Nickel-nitrilotriacetic acid
nm	Nanometers
nM	Nanomolars
NTP	Ribonucleoside triphosphate
OD_{600}	Optical Density at 600 nm
ONPG	2-Nitro-phenyl-β-D-galactopyranoside
ORF	Open reading frame
oriT	Origin of transfer
oriV	Origin of replication
PCR	Polymerase chain reactions
P_{finP}	Promoter for *finP*

P_{int}	Internal *traM* promoters
P_{lac}	Promoter for the *lac* operon
P_{traJ}	Promoter for *traJ*
P_{traM}	Promoters for *traM*
P_{traY}	Promoter for *traY*
PVDF	Polyvinylidene difluoride
R	Resistant
RBS	Ribosome binding site
rpm	Revolutions per minute
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
Spc	Spectinomycin
ss	Single strand
Str	Streptomycin
T	Thymine
Tc	Tetracycline
tra	Transfer
Tris	Tris (hydroxymethyl) aminomethane
T_{traJ}	Transcriptional terminator for *traJ*
T_{traM}	Transcriptional terminator for *traM*
UTR	Untranslated region
UV	Ultraviolet
V	Volts
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
µl	Microliters
µg	Micrograms
°C	Degree Celsius

Chapter 1

General Introduction

1.1 Horizontal DNA transfer

Horizontal gene transfer allows bacteria to acquire new genes to exploit new environments and respond to selective pressure (Syvanen, 1994). Comparison of the *Escherichia coli* and *Salmonella enterica* genomes suggests that approximately 17.6% of the genes present in the *E. coli* genome have been acquired by horizontal gene transfer (Lawrence and Ochman, 1998).

Horizontal DNA transfer occurs intracellularly and intercellularly. Intracellular DNA transfer is accomplished by means of non-conjugative transposition, in which defined DNA elements move within a genome, or by recombination, in which DNA fragments crossover into another location in a genome. Intercellular DNA transfer includes transformation, in which cells take up DNA directly from their environment; transduction, in which a phage injects host DNA from one bacterium into another; and conjugation, in which conjugative plasmids (or conjugative transposons) transfer or are mobilized from donor to recipient cells.

Intracellular DNA transfer can reorganize or change genetic traits of host cells, allowing cells to adapt to the continually changing environment and evolve as directed by natural selection. Intercellular DNA transfer enables cells to acquire new traits from a greater variety of genetic sources and provides a convenient and efficient way for cells to exploit new habitats or to evolve after accumulation of enough foreign DNA.

1.2 Conjugative transfer of DNA

1.2.1 General properties

Transformation and transduction mediate DNA transfer at very low frequencies, whereas conjugation transports DNA at high frequencies through direct contact between cells that can belong to different species, different genera or different kingdoms (Zupan and Zambryski, 1995; Bundock and Hooykaas, 1996; Waters, 2001). Therefore, conjugative DNA transfer has a greater genetic and evolutionary significance than transformation and transduction. Most plasmids in bacteria are either self-transmissible or mobilizable, suggesting that conjugation is advantageous for plasmids and their hosts.

Conjugation is a specialized process involving unidirectional transfer of a single-stranded DNA from a donor to a recipient cell by a mechanism that requires cell-cell contact. This process is multi-step and relies on a specific DNA sequence (*oriT*, origin of transfer), and many proteins that are involved in formation of a cytoplasmic nucleoprotein complex and a membrane-bound DNA transfer apparatus (Lanka and Wilkins, 1995; Llosa *et al.*, 2002). An *oriT* region contains a *nic* site (where the transferred strand is cleaved) and multiple binding sites for DNA-processing proteins. DNA-processing proteins bind to *oriT* to form a nucleoprotein macromolecular structure called the relaxosome (Clewell and Helinski, 1969). Based on sequence similarity at the *nic* site, plasmids are categorized into five families, which are exemplified by F, RP4, RSF1010, ColE1 and pMV158 (Lanka and Wilkins, 1995; Guzman and Espinosa, 1997; Zechner *et al.*, 2000). The most studied protein involved in formation of a relaxosome is called the relaxase that has either nickase or nickase and DNA helicase activity (Byrd and Matson, 1997). The relaxase introduces a single-stranded nick at the *nic* site and

covalently attaches to the 5' end of the nicked strand that will be transferred into the recipient cell.

Conjugative transfer needs formation of a stable mating pair between a donor and a recipient cell (Kingsman and Willetts, 1978). The pairing area presumably contains the DNA transport machinery (transferosome) but its detailed structure remains unknown. The relaxosome is probably anchored through interactions with the transferosome with a single DNA strand transferred in a 5' to 3' direction from the donor to the recipient (Willetts and Wilkins, 1984; Lanka and Wilkins, 1995). The single strand is replicated by replacement strand synthesis in the donor cell and by complementary strand synthesis in the recipient cell (Willetts and Wilkins, 1984). The conjugative DNA transport machinery belongs to the type IV secretion system, which also includes some specialized protein transport systems such as the Dot/Icm system in *Legionella pneumophila* (Zechner *et al.*, 2000; Christie and Vogel, 2000). The Dot/Icm system, which transports effector proteins, can also transfer mobilizable plasmid RSF1010 to *E. coli* strains (Segal *et al.*, 1998; Vogel *et al.*, 1998), suggesting a close relation between DNA transfer and protein transport systems. The DNA transfer machinery goes through both the donor and the recipient cell envelopes and involves the Mpf (mating pair formation) proteins. All transfer systems in gram-negative organisms need a sex pilus synthesis for function. Based on sex pilus morphology and pilus-specific phage sensitivity, the pilus-dependent transfer systems belong to two evolutionary families exemplified by F and RP4 respectively (Frost, 1993). Conjugation in gram-positive organisms does not appear to be dependent on pili, but pheromones or clumping-inducing agents are needed for efficient conjugation (Clewell, 1993; Dunny *et al.*, 1995).

1.2.2 Conjugative plasmids and transposons

Self-transmissible plasmids can transfer among different hosts, and sometimes aid in transfer of chromosomal DNA and mobilizable plasmids. The most well studied self-transmissible plasmids belong to IncF, IncI, IncN, IncP and IncW incompatibility groups. There are also many plasmids that are smaller and not self-transmissible but contain *oriT* and gene(s) encoding all the DNA processing proteins. These plasmids can utilize a compatible transferosome provided by a co-resident self-transmissible plasmid and conjugatively transfer to other cells. Therefore, these plasmids are called mobilizable plasmids, with ColE1, pSC101 and IncQ plasmids being among the best known.

Conjugative transposons are transposons combined with conjugative transfer functions. Although plasmids are believed to mediate the majority of gene transfer events, conjugative transposons have been recognized as important vehicles of genetic exchange (Salyers *et al.*, 1995; Scott and Churchward, 1995). Tn*916* is the best-studied example of the conjugative transposons (Gawron-Burke and Clewell, 1982; Scott and Churchward, 1995). It can excise from donor genomes or plasmids and conjugatively transfer to recipient cells, where they can integrate into recipient genomes to be replicated. Mobilizable transposons are similar to mobilizable plasmids in that they are small and not self-transmissible but contain *oriT* and gene(s) encoding all the DNA processing proteins. Genetic elements in Bacteroides such as the β-lactamase transposon Tn*4555* (Parker and Smith, 1993), cryptic Tn*4399* (Hecht and Malamy, 1989), Tn*5520* (Vedantam *et al.*, 1999), and non-replicating Bacteroides units (NBUs) (Shoemaker and Salyers, 1988) are among a few characterized elements in this group. To a great degree,

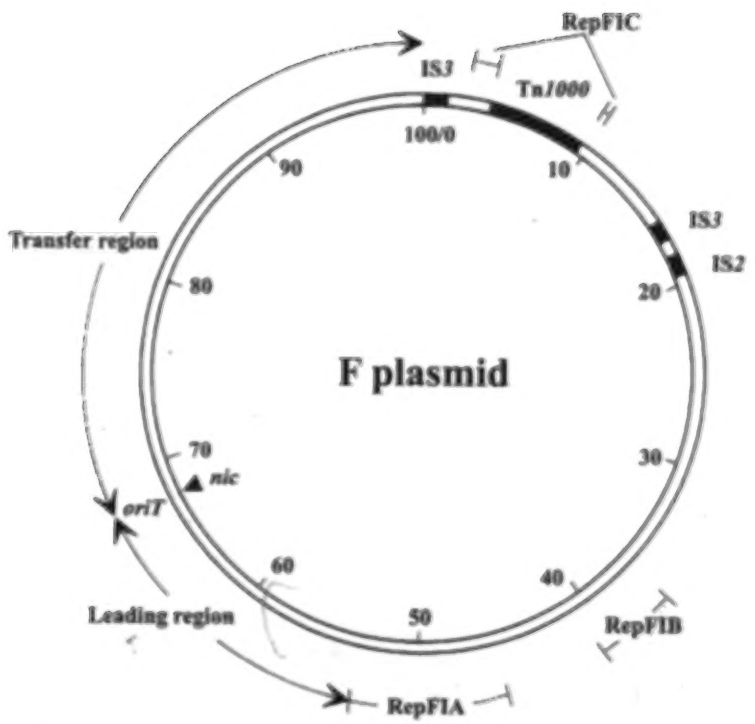
plasmids and conjugative transposons share many common properties. Mobilizable plasmids can be mobilized by self-transmissible transposons and mobilizable transposons can be mobilized by self-transmissible plasmids (Vedantam *et al.*, 1999).

1.3 Molecular genetics of the F plasmid

1.3.1 General properties

The F plasmid is a 100-kb circular plasmid in the IncFI incompatibility group (Figure 1-1). It was first discovered in *Escherichia coli* K-12 as an "infectious vector" in 1946 (Lederberg and Tatum, 1946) and then named the fertility (F) factor (Hayes, 1953a). The F plasmid can replicate and transfer among many other enteric bacteria including *Salmonella typhimurium* (Zinder, 1960). It also can integrate into the host chromosome via its insertion sequences (IS*2* and IS*3*) to cause the transfer of the host chromosome, leading to formation of F-prime factors or high frequency recombination (Hfr) in the recipient cells (Heyes, 1953b; Adelberg and Burns, 1960; Deonier and Hadley, 1980; Umeda and Ohtsubo, 1989). F^+ bacteria are characterized by their thick, flexible pili (Bradley, 1980) and by their sensitivity to F-specific bacteriophages (Frost, 1993).

A large group of conjugative plasmids found throughout the family *Enterobacteriaceae* are known collectively as "F-like plasmids" due to the similarity of the pili they express and the bacteriophage sensitivities they confer (Lawn *et al.*, 1967; Datta, 1975; Jacob *et al.*, 1977). Based on plasmid incompatibility, these plasmids can be further grouped into seven incompatibility groups from IncFI to IncFVII (Datta, 1975; Jacob *et al.*, 1977; Ippen-Ihler and Skurray, 1993), which are collectively called the IncF complex. Many plasmids in the IncF complex encode a range of ecologically and


RepFIC
IS3
Tn1000
100/0
10
90
IS3
IS2
Transfer region
80
20
F plasmid
70
30
nic
oriT
60
40
Leading region
50
RepFIB
RepFIA

medically important properties, such as antibiotics resistance and toxin production, which are disseminated by way of bacteria conjugation (Ippen-Ihler and Skurray, 1993).

1.3.2 Plasmid replication

Vegetative replication of the F plasmid follows the theta-type model (del Solar *et al.*, 1998). During replication, a bubble is formed between the separated DNA strands at the site of replication initiation. The bubble grows as replication continues, forming a theta-like plasmid DNA with the replication "fork" at the site(s) of synthesis. The F plasmid contains three replicons including RepFIA, RepFIB, and RepFIC that is inactivated by Tn*1000* insertion (Figure 1-1; Willets and Skurray, 1987; Couturier *et al.*, 1988). The RepFIA (44.6 kb to 53.7 kb in the F map) is the primary replicon, which possesses all necessary functions for maintenance of the F plasmid (Lane, 1981; Kline, 1985), whereas RepFIB (38.0 kb to 39.9 kb in the F map) is the secondary replicon, which is inherently unstable (Lane and Gardner, 1979; Lane, 1981). RepFIA contains both unidirectional (*oriS* or *ori2*, around 49.4 kb in the F map) and bi-directional (*oriV* or *ori1*, around 46.9 kb in the F map) origins of replication, and encodes proteins responsible for copy number control, active partitioning, and incompatibility (Willetts and Skurray, 1987). The basic origin of replication for the RepFIA replicon is *oriS*, which mimics F replication, whereas removal of *oriV* does not affect the stabilization of the F plasmid (Manis and Kline, 1977; Eichenlaub *et al.*, 1981).

The 217-bp *oriS* contains two DnaA boxes (TTATCCAC), a 46-bp A/T rich region, a 13mer sequence, and four 19-bp direct repeats (iterons) (Murotsu *et al.*, 1981; Trawick and Kline, 1985). Immediately downstream of *oriS* is *repE* (49.46 kb to 50.03 kb in F)

along with *incC*, *sopA*, *sopB* and *sopC*. (Figure 1-2). The initiation of F replication follows the iteron-binding model (Chattoraj, 2000). RepE monomers bind to four iterons in *oriS* and cause bending of this region, which in turn induces localized melting of duplex DNA from the 13mer with the assistance of HU (heat-unstable nucleoid protein). DnaA extends melting to the AT-rich region, which serve as an entry for DnaB-DnaC helicase through protein-protein interactions (Kawasaki *et al.*, 1996). Following the helicase, DnaG (primase) and DnaE (DNA polymerase) enter the denatured area in order and DNA replication starts (del Solar *et al.*, 1998).

Besides vegetative replication, the F plasmid also undergoes conjugative DNA synthesis during conjugation, which includes replacement strand and complementary strand synthesis in the donor and recipient cells, respectively (Willetts and Wilkins, 1984; Wilkins and Lanka, 1993). Conjugation can be viewed as a replicative process that ensures plasmid maintenance in the donor and introduces a new plasmid molecule into the recipient (Waters, 1999). The site-specific and strand-specific nicking at *oriT* is very similar to the events initiating rolling-circle replication, the unidirectional DNA replication mechanism accomplished by extension of the nicked strand at the 3' OH end (del Solar *et al.*, 1998; Waters and Guiney, 1993). The replacement strand synthesis in the donor cell and complementary strand synthesis in the recipient cell also match the two stages of rolling-circle replication exemplified by pT181 (Birch and Khan, 1992). In the IncQ plasmid R1162, the 3' end of the nicked strand is elongated by DNA synthesis to produce a greater-than-unit plasmid DNA during conjugation, supporting the rolling-circle model for conjugative DNA synthesis (Erickson and Meyer, 1993). However, rolling-circle replication might not be the only mechanism for replacement strand

synthesis in R1162 since conjugative DNA synthesis in donors occurs with a lag between rounds of transfer and with most of the DNA synthesis requiring the origin for vegetative replication (Parker and Meyer, 2002; Parker *et al.*, 2002). Therefore, the mechanism for conjugative DNA synthesis requires further investigation.

1.3.3 Plasmid stabilization

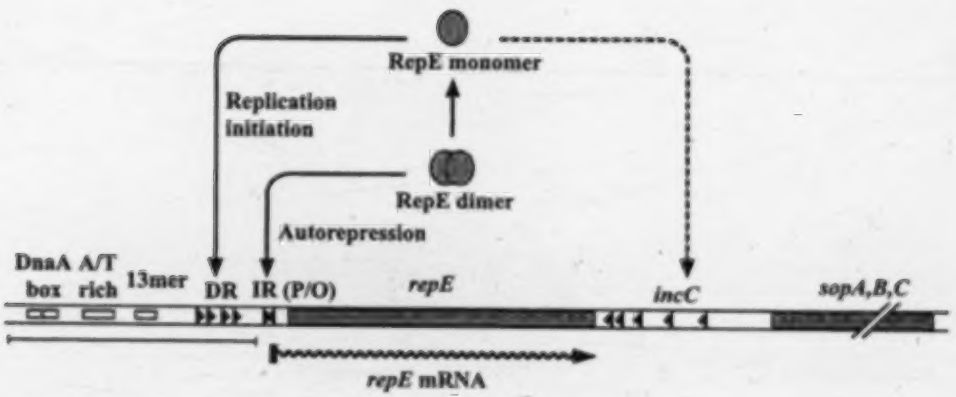
One of the most distinctive features of the F plasmid is its ability to maintain a copy number of 1 or 2 per chromosome (Frame and Bishop, 1971). This finely tuned control of plasmid stability is realized by coordination of replication, copy number control and partitioning, involving a number of genes in the host chromosome or the F plasmid.

The F plasmid has at least two mechanisms of regulating replication initiation that control its copy number (Figure 1-2). Firstly, RepE regulates its own expression at the transcriptional level (Trawick and Kline 1985; Wada *et al.*, 1987). RepE monomers are active for replication initiation but inactive for autoregulation, whereas RepE dimers are active for autoregulation but inactive for replication initiation (Ishiai *et al.*, 1994). When RepE monomers increase, RepE dimers also increase and in turn stop a further increase of RepE expression. By this feedback inhibition mechanism, the concentration of RepE monomers, which is proportional to the frequency of initiation of DNA replication, is stably maintained. Mutations in *repE* can cause variance in plasmid copy number, and thus *repE* is also named *copA* (Trawick and Kline, 1985).

Another mechanism involves *incC*, which contains five directly repeated iterons identical to those of *oriS* but oriented in the opposite direction (Kline and Trawick, 1983). A titration model has been proposed whereby binding of RepE to the *incC* iterons

Figure 1-2. Genetic organization of *oriS* and replication control by RepE and *incC*.

DR: 19-bp direct repeat; IR: inverted repeat in the *repE* promoter; A/T: A/T rich region (modified from Uga *et al.*, 1999).


RepE monomer
Replication initiation
RepE dimer
Autorepression
DnaA box
A/T rich
13mer
DR
IR (P/O)
repE
incC
sopA,B,C
repE mRNA

represses the initiation of DNA replication by limiting the supply of RepE to the replication origin (Tsutsui *et al.*, 1983). When the plasmid copy number increases, the increased *incC* sites will compete with the iterons at *oriS*, resulting in reduced replication initiation and reduced plasmid copy number, explaining why *incC* is also called *copB* (Kline, 1979; Seelke *et al.*, 1982; Kline, 1985). The same model applies to the function of *incC* in determining plasmid incompatibility, explaining the name *incC*. However, the titration model has been questioned by a report that initiation of mini-F DNA replication is inhibited by formation of a nucleoprotein complex consisting of *oriS* iterons and *incC* iterons bound by RepE (Uga *et al.*, 1999). The new model suggests that the copy number of the F plasmid is regulated by a cross-linked structure (handcuffing). Because RepE monomers bind to *ori2* approximately 1.5 to 3 times more efficiently than to *incC*, RepE functions as a replication initiator at a comparatively low concentration. When plasmid copy number increases, RepE monomers increase accordingly thereby being able to bind to *incC*. The *ori2-incC* interaction, which is mediated by RepE dimerization, thus occurs. This interaction can be an *in trans* interaction (intermolecular) or an *in cis* interaction (intramolecular). In both types of interactions, the replicative origin is tethered to prevent further replication initiation. This replication initiator-mediated handcuffing model for regulating plasmid copy number has been suggested to be common in plasmids that have an iteron-binding process during replication initiation (Chattoraj, 2000).

Besides *incC*-related copy number control, the *sopABC* partitioning system also contributes to F plasmid stability (Hiraga, 2000). SopA, SopB, and a *cis*-acting *sopC* site that contains twelve 43-bp direct repeats are essential for partitioning (Ogura and Hiraga, 1983; Mori *et al.*, 1986). SopA binds to SopB, whereas SopB binds to *sopC* (Hanai *et al.*,

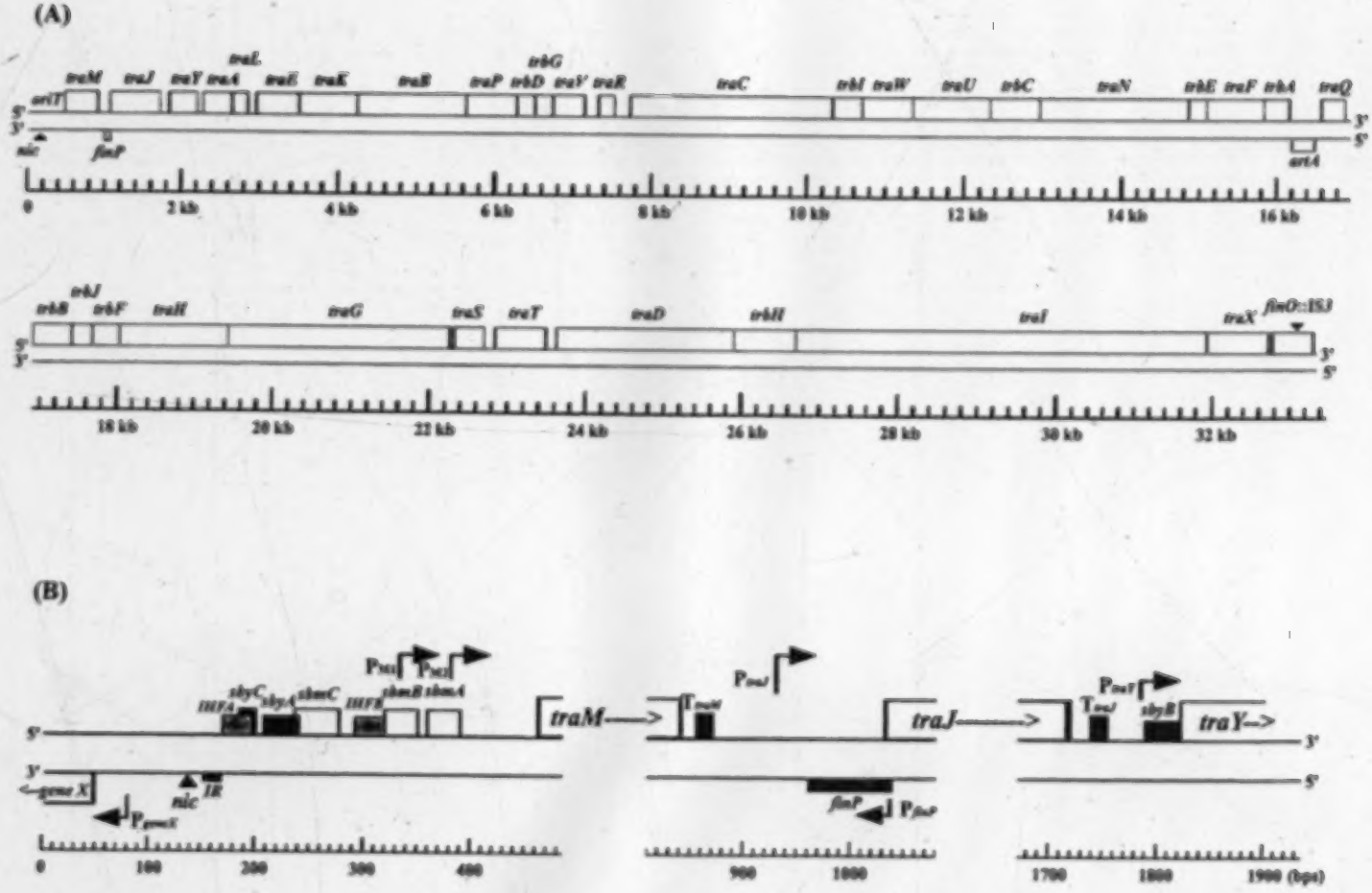
1996; Kim and Shim, 1999). It has been proposed (Austin and Nordström, 1990) that plasmid-encoded partitioning proteins bind specifically to a *cis*-acting site and promote pairing of plasmid DNA molecules via dimerization of the proteins. The paired protein–plasmid DNA complexes associate with specific cellular site(s) at the future septation plane. The septum is formed between the paired complexes, so the complexes are separated from each other by the completion of septation and partitioning into the daughter cells. Observations that segregated F plasmids migrate rapidly to the middle of the daughter cells during cell division further supports this model (Niki and Hiraga, 1997; Gordon *et al.*, 1997). For the IncFII plasmid R1, the *parC* (equivalent to *sopC*)/ParR (equivalent to SopB) complex causes ParM (equivalent to SopA) to form actin-like filaments to push the replicated plasmids apart; however, the mechanism for SopA, SopB, and *sopC* function in F plasmid partitioning remains unclear (Gerdes *et al.*, 2004).

1.3.4 F Conjugation

1.3.4.1 Organization of the F *tra* region

The 33.3-kb *tra* region maps at 66.7 kb to 100 kb in the F plasmid, and contains 38 open reading frames, of which 28 are involved in conjugation with 20 being essential (Frost *et al.*, 1994). F *oriT* maps at the upper end of the transfer region and extends approximately 250 base pairs with *traM*, *traJ* and the major *tra* operon containing the rest of the *tra* genes aligned downstream in order (Figure 1-3 A). Immediately upstream of the *tra* region is the 13-kb leading region (53.3 kb to 66.7 kb in F), which is the first to enter recipient cells during conjugation and which encodes proteins that assist in establishing F DNA in the recipients (Ray and Skurray, 1983; Firth *et al.*, 1996).

Figure 1-3. Diagram of the F transfer region. The coordinates are the same as in Frost *et al.* (1994). The triangle under the lower strand indicates the *nic* site. (A) Complete F *tra* region. Clear boxes represent open reading frames (ORFs) except for *finP* that encodes an anti-sense RNA. The triangle above *finO* indicates the site of IS2 insertion. (B) Region from *oriT* to the beginning of *traY*. Clear and black boxes represent protein-binding sites, sequence motifs, or genes. Open boxes represent incomplete genes with arrows indicating their orientations. Angled arrows indicate the location and the direction of promoters. P_{m1} and P_{m2} are the two *traM* promoters collectively called P_{traM}. The abbreviations used are list in the "Abbreviations" section.


(A)
traM traJ traY traA traL traE traK traB traP trbD traV traR traC trbI traW traU trbC traN trbE traF trbA traQ
oriT
nic
finP
ort4
0 2 kb 4 kb 6 kb 8 kb 10 kb 12 kb 14 kb 16 kb
trbJ
trbB trbF traH traG traS traT traD trbH traI traX finO::IS1
18 kb 20 kb 22 kb 24 kb 26 kb 28 kb 30 kb 32 kb
(B)
IHFA sbyC sbyA sbmC IHFB sbmB sbmA
traM traJ traY
sbyB
orfx X
nic IR
finP
0 100 200 300 400 900 1000 1700 1800 1900 (bps)

The sequence between 100-bp upstream (5') and 40-bp downstream (3') of *nic* is conserved (Thompson *et al.*, 1989; Fu *et al.*, 1991; Gao *et al.*, 1994). The inverted repeat immediately upstream of *nic* (towards the transfer region) is important for the relaxase-catalyzed termination of transfer (Fu *et al.*, 1991). Similar repeats in R1162 *oriT* are also important for binding of relaxase and termination of a round of transfer (Bhattacharjee *et al.*, 1992; Bhattacharjee and Meyer, 1993). Further upstream (5') of *nic* are binding sites for integration host factor (IHFA and IHFB) and F-encoded TraY and TraM (Tsai *et al.*, 1990). Two TraY binding sites have been identified in F *oriT* with *sbyC* overlapping an IHF binding site (IHFA) near *nic*, and *sbyA* partially overlapping a TraM binding site (Luo *et al.*, 1994; Lahue and Matson, 1990). There are three TraM binding sites in *oriT* including two sites with highest affinity (*sbmA* and *sbmB*) overlapping the two *traM* promoters (collectively called P_{traM}) and another site with the lowest affinity (*sbmC*) overlapping part of *sbyA* (Di Laurenzio *et al.*, 1992). Deletion analysis has indicated that the left half of *sbyA* and the IHFA-binding site are required for nicking, whereas *sbyA*, IHFA and *sbmC* are required for transfer (Fu *et al.*, 1991).

1.3.4.2 Gene regulation and relaxosome formation

Proteins encoded by the F *tra* region perform conjugation-related functions including regulation of *tra* gene expression and formation of the relaxosome and transferosome. Regulation of *tra* gene expression and formation of the relaxosome are tightly related events during DNA transfer. In the F plasmid, genes such as *traM* and *traY* participate in both these processes (Penfold *et al.*, 1996, Taki *et al.*, 1998; Fekete and Frost, 2000), suggesting that regulation and formation of the relaxosome are coordinated

in some way. An extreme example is that of R1162, where formation of the relaxosome is required for regulation of *mob* gene expression (Perwez and Meyer, 1999; Zhang *et al.*, 2003).

In the F and most F-like plasmids, *traM*, *traJ*, *traY*, *finO*, and *finP* are involved in regulation of *tra* gene expression. The central switch for *tra* gene regulation, *traJ*, is located between *traM* and *traY* (the first gene in the major *tra* operon) and encodes a positive regulator (TraJ) required by P_{traY}, the promoter for the major *tra* operon (Fowler *et al.*, 1983; Gaffney *et al.*, 1983; Willetts, 1977; Figure 1-3 B). In R100 (an IncFII plasmid), the TraJ binding site (*sbj*) is 93 bp upstream of P_{traY} and presumably contacts RNA polymerase to promote transcription of the major *tra* operon (Taki *et al.*, 1998). FinP, an anti-sense RNA complementary to the 5' non-translated region (5' UTR) of *traJ* mRNA, is encoded by *finP* that is located on the complementary strand (Figure 1-3 B; Frost *et al.*, 1994). FinO, a protein encoded by *finO* at the 3' end of major *tra* operon, binds to FinP through its N-terminal flexible region to increase FinP-*traJ* mRNA binding and protect FinP from RNase E-mediated degradation (Biesen and Frost, 1994; Jerome *et al.*, 1999; Ghetu *et al.*, 2000; Arthur *et al.*, 2003). By blocking the ribosome binding site of *traJ* mRNA, the FinOP complex stringently represses TraJ translation, and thus represses expression of the major *tra* operon (van Biesen and Frost, 1994; Koraimann *et al.*, 1996). However, *finO* is disrupted by an IS*3* element in the F plasmid, allowing the F plasmid to transfer at a frequency at least 100-fold higher than most other F-like plasmids (Cheah and Skurray, 1986; Yoshioka *et al.*, 1987; Figure 1-3 A).

TraY binds to DNA as a monomer or a dimer (Lahue and Matson, 1990; Nelson and Matson, 1996). TraY binds to *sbyB* at P_{traY} as well as to *sbyA and sbyC* at *oriT* (Nelson *et*

al., 1993), allowing TraY to regulate *traM* and its own transcription (Penfold *et al.*, 1996; Maneewannakul *et al.*, 1996). TraY is a positive regulator for both P_{traM} and P_{traY} in the F plasmid (Silverman and Sholl, 1996; Penfold *et al.*, 1996), whereas in R100, TraY acts as a negative regulator of P_{traY} (Taki *et al.*, 1998). As relaxosome components, TraY together with IHF probably cause a structural change in the DNA that facilitates the binding of TraI (relaxase) to the *oriT* region and consequent nicking (Tsai *et al.*, 1990; Luo *et al.*, 1994; Nelson *et al.*, 1995; Fekete and Frost, 2000).

TraM has been suggested to bind to specific DNA sites as tetramers (Verdino *et al.*, 1999; Miller and Schildbach, 2003). TraM negatively regulates its own expression at the transcriptional level by binding to sites overlapping the *traM* promoters (Di Laurenzio *et al.* 1992; Penfold *et al.*, 1996). In R1 and R100, *traM* also positively regulates transfer gene expression by readthrough transcription from one of the *traM* promoters for R1 (Koraimann *et al.*, 1996; Polzleitner *et al.*, 1997), or from a putative promoter inside *traM* for R100 (Dempsey, 1994; Stockwell and Dempsey, 1997). TraM is essential for F conjugation but is not required for F-pilus assembly or mating pair formation (Willetts and Wilkins, 1984). TraM is a component of the relaxosome, but it is not important for the nicking reaction catalyzed by TraI in the presence of IHF and TraY (Howard *et al.*, 1995; Nelson *et al.*, 1995; Frost and Fekete, 2000). Since TraM also binds to the coupling protein TraD (Disque-Kochem and Dreiseikelmann, 1997), TraM could be the key protein that mediates interaction between the relaxosome and the membrane transfer apparatus, which agrees with the previous proposal that TraM functions as a signaling factor during F conjugation (Willetts and Wilkins, 1984).

TraI, the relaxase, was first characterized as DNA helicase I and later was found to be the protein that cleaves DNA at the *nic* site (Abdel-Monem *et al.*, 1976; Traxler and Minkley, 1988). The N -terminus and C-terminus of TraI have transesterase (relaxase) and helicase activities, respectively, with both being essential for F conjugation (Reygers *et al.*, 1991; Matson *et al.*, 2001; Byrd *et al.*, 2002). TraI-catalyzed cleavage is not only site-specific but also strand-specific, resulting in only one of the strands (the lower strand in Figure 1-3) being nicked (Matson and Morton, 1991). The nicking reaction involves a reversible transesterification between the 5' phosphate of a guanosyl residue on the 3' side of *nic* and a tyrosine near the N-terminal end of the protein (Byrd and Matson, 1997; Matson *et al.*, 2001). After nicking, the 5' end of the nick strand is covalently linked to TraI (Matson *et al.*, 1993), whereas the 3' end of the DNA is non-covalently attached to TraI, forming a cytoplasmic nucleoprotein structure (relaxosome) together with IHF, TraM, and TraY. During DNA transfer, TraI might use its helicase activity to catalyze unwinding and thus providing the force to drive a single strand of DNA from the donor to the recipient cell. The relaxase of most other plasmid transfer systems does not have helicase activity; they have been suggested to recruit either a host- or plasmid-encoded helicase (Lanka and Wilkins, 1995).

1.3.4.3 Formation of the membrane transfer apparatus

F-pili assembly and retraction are required to establish cell–cell contacts but not required for the following DNA transfer process during conjugation (Panicker and Minkley, 1985; Firth *et al.*, 1996). F pili are assembled from pilin that is processed from propilin encoded by *traA* in the major *tra* operon (Moore *et al.*, 1981). Propilin is inserted

in the inner membrane through the action of TraQ, an F-pilin-specific chaperone that interacts with F pilin (Maneewannakul *et al.*, 1993; Majdalani and Ippen-Ihler, 1996; Paiva and Silverman, 1996; Harris *et al.*, 1999). Propilin is cleaved to pilin by host leader peptidase and is acetylated at its N terminus by TraX (Moore *et al.*, 1993). Mature pilin is assembled into functional pilus filaments by more than a dozen other *tra* gene products (Frost *et al.*, 1984; Penfold *et al.*, 1994; Schandel *et al.*, 1992; Firth and Skurray, 1992; Frost *et al.*, 1994; Anthony *et al.*, 1999; Harris *et al.*, 2001). It is not clear which proteins are responsible for pilus retraction, with only TrbI thought to be a candidate (Maneewannakul *et al.*, 1992).

TraN and TraG participate in mating pair stabilization, which allows F^+ cells to mate more efficiently in liquid media and to resist disaggregation by shear forces or the addition of chaotropic agents (Achtman *et al.*, 1972; Miki *et al.*, 1978; Manning *et al.*, 1981). TraN is an outer membrane protein and has been suggested to interact with OmpA and lipopolysaccharide moieties in the recipient cell (Maneewannakul *et al.*, 1992; Klimke and Frost, 1998). TraG is an inner membrane protein with it N-terminal region being homologous to VirB6 of the Ti plasmid (Lawley *et al.*, 2003). An interaction between periplasmic domains of TraN and TraG might be needed to form a stable and functional connection between conjugating cells (Durrenberger *et al.*, 1991, Firth *et al.*, 1996).

TraD is a member of the "coupling protein" family that has been proposed to couple the relaxosome to the transport site during conjugation (Cabezón, *et al.*, 1997). This agrees with the observation that TraD is essential for conjugation but functions after other transfer-related stages such as mating pair formation and DNA processing (Everett and

Willetts, 1980; Kingsman and Willetts, 1978; Panicker and Minkley, 1985). TraD is an inner membrane protein with two membrane spanning regions and the amino- and carboxyl-terminal regions in the cytoplasm (Lee *et al.*, 1999). TraD can bind to DNA non-specifically (Panicker and Minkley, 1992). The carboxyl-terminal domain of TraD is important for mobilization of F-specific plasmids; its removal increases mobilization of some plasmids but decreases mobilization of F-specific plasmids (Sastre *et al.*, 1998). TraD interacts with TraM (Disque-Kochem and Dreiseikelmann, 1997) as well as TraI when TraI and TraD are both over-expressed (Dash *et al.*, 1992), further suggesting that the relaxosome may be anchored to the membrane-bound transfer machinery through specific interactions between TraD and the relaxosome components (Frost *et al.*, 1994). Coupling proteins share sequence similarities with some known DNA pumps, suggesting that coupling proteins might also serve as a pump to push the single strand DNA through the membrane passage once the relaxosome is anchored to the transferosome (Llosa *et al.*, 2002). This hypothesis agrees with the crystal structure of the C-terminal cytoplasmic portion of TrwB (the coupling protein of R388) that resembles ring helicases and F_1-ATPase (Gomis-Ruth *et al.*, 2001).

1.4. Objectives

One objective of this work was to elucidate the ambiguous functions of TraM in F conjugation and understand how the structure of TraM contributes to these functions. TraM forms tetramers, binds to cognate DNA, and interacts with TraD (Di Laurenzio *et al.*, 1992; Disque-Kochem and Dreiseikelmann, 1997; Miller and Schildbach, 2003). However, whether and how these properties of TraM contribute to autoregulation and F

conjugation remain unknown. Different screens were developed to select TraM mutants that are defective in autoregulation or F conjugation. Selected mutants were characterized for oligomerization, cognate DNA binding, and interactions with TraD. Domains for oligomerization, DNA binding, and interactions with TraD could be determined by correlating defects of TraM mutants with the locations of corresponding mutations. A model is proposed to demonstrate how tetramerization, DNA binding, and interactions with TraD contribute to TraM in autoregulation and F conjugation.

The other objective of this work was to understand how *traM* (or TraM) participates in *tra* gene regulation. The effect of TraM overexpression on host cells was investigated to understand why *traM* autoregulation is so common in F-like plasmids (Abo and Ohtsubo, 1993; Schwab *et al.*, 1993; Penfold *et al.*, 1996). Possible transcriptional readthrough from *traM* into *traJ* was also determined using a promoter assessment plasmid. The necessity for strong P_{traM} in the F plasmid was examined by measuring F conjugation in new transconjugants or when host cells are recovering from stationary phase. When F^+ cells are in stationary phase, TraM and TraY are not detectable (Frost and Manchak, 1998), which brought up a question about the regulation of P_{traM}. TraY-dependent TraM transcription could be responsible (Penfold *et al.*, 1996), however the mechanism was obscure. By integrating results from this project and previous studies, a model is proposed that explains how *traM* fits into the circuit of *tra* gene regulation.

Chapter 2

Materials and Methods

2.1 Growth media and bacterial strains

Cells were grown in LB (Luria-Bertani) broth or on LB solid medium containing appropriate antibiotics or other supplements (Sambrook *et al.*, 1989). Lactose-based MacConkey agar (Difco) plates were used to detect cells carrying F_0*lac* or its derivatives. Antibiotics were used at the following final concentrations: ampicillin (Amp), 50 μg/ml; kanamycin (Km), 25 μg/ml; streptomycin (Str), 200μg/ml; spectinomycin (Spc), 100 μg/ml; nalidixic acid (Nal), 40 μg/ml; chloramphenicol (Cm), 50 μg/ml; and tetracycline (Tet), 10 μg/ml. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, BCIG) was used at a final concentration of 100 μg/ml. IPTG (isopropylthio-β-D-galactoside) was used at a final concentration of 1 mM. The following *Escherichia coli* strains were used: XK1200 [F⁻ Δ*lacU124* Δ*(nadA gal attλ bio) gyrA* (Nal^r); Moore *et al.*, 1987], ED24 (F⁻ Lac⁻Spc^r; Willetts and Finnegan, 1970), DH5α [Δ*lacU169 (Φ80 lacZΔM15) supE44 hsdR17 recA1 endA1 gyrA96* (Nal^r) *thi-1 relA1*; Hanahan, 1983], and BL21-DE3 [F⁻ *dcm ompT hsdS*(r_B^- m_B^-) *gal* λ(DE3), Stratagene™].

2.2 DNA manipulation, PCR, DNA sequencing, and sequence analysis

DNA purification, manipulation, and PCR (polymerase chain reaction) followed standard procedures (Sambrook *et al.*, 1989) or protocols from the manufacturers. Klenow fragment (Roche Diagnostics) was used to blunt digested DNA fragments. Vent DNA polymerase (New England BioLabs) was used for all PCR reactions except for error-prone PCR, which used *Taq* DNA polymerase. Miniprep and Gel Extraction Kits (Qiagen) were used for plasmid purification and extraction of DNA fragments from agarose gels, respectively. DNA sequencing was performed using the Amersham

DYEnamic™ ET terminator cycle sequencing kit and an Applied Biosystems 373-S DNA Sequencer with XL upgrade. DNA and protein sequences were compiled and analyzed using Genetool® and Peptool® software. General homology searches were performed using BLAST provided by NCBI (www.ncbi.nlm.nih.gov). Promoters were determined using the Neural Network Promoter Predictor (www.fruitfly.org) for prokaryotic promoters based on similarity to the $E\sigma^{70}$ consensus and the logic of gene organization.

2.3 Oligonucleotides, bacterial plasmids, and plasmid construction

Oligonucleotides were synthesized on an Applied Biosystems 392 DNA synthesizer in the Department of Biological Sciences. The plasmids and oligonucleotides (not including primers for sequencing the pED208 transfer region) used in this work are listed in Table 2-1. The DNA sequences of the F plasmid, the pED208 *tra* region, pPR9tt, pBR322, and pBluescript KS+ are under GenBank® accession numbers U01159, AF411480, AF187996, J017490, and X52327, respectively.

An 8-kb *Xho*I-*Hin*dIII fragment from pBF101 was cloned in pBluescript KS+, resulting in pJLED1. A 0.65-kb *Bst*BI (blunted)-*Sac*I fragment from pNY300 was cloned into the *Eco*RI (blunted)-*Sac*I sites of pT7-5, resulting in pRFM200, which contains *traM* and the downstream *traJ* promoter (P_{traJ}). The *Sma*I-digested fragment of pUC118 was ligated to a 0.4-kb DNA fragment amplified from pNY300 using RFE6 and SPE9 as primers. The 0.4-kb *Bam*HI-*Kpn*I fragment (containing *traM*) from the resulting plasmid was cloned in *Bam*HI-*Kpn*I sites of pQE40 (Qiagen) to form pRF400. pRF402 is identical to pRF400 except that it contained *traM*I109T (the 109 codon of *traM* was mutated from

isoleucine to threonine) instead of wild type *traM* due to a PCR incorporation error. The 0.3-kb *Sal*I-*Sal*I fragment from pRF400 or pRF402 replaced the 0.5-kb *Sal*I-*Sal*I fragment in pRFM200 such that *traM* was not disrupted, resulting in pJLM001 or pJLM002, respectively. The 2.8-kb *Sma*I (blunt)-*Hin*dIII fragment from pJLM002 was ligated to the 0.25-kb *Hin*dIII-digested DNA fragment amplified from pRFM200 using JLU2 and JLU4 as primers such that P_{traJ} is in the same orientation as in pRFM200, resulting in pJLM003. pJLM004 was constructed by ligating the 2.4-kp *Eco*RI-*Kpn*I fragment of pRFM200 with the 0.6-kb *Eco*RI-*Kpn*I fragment of DNA amplified from pRFM200 using JLU75 and JLU76 as primers. The first 108 codons of *traM* in pRFM200 or pJLM004 were deleted by religation of the 2.7-kb *Eco*RI (blunted) -*Eco*RV (blunt) fragments of pRFM200 or pJLM004, resulting in pRFM200-Mdel and pJLM004-Mdel, respectively. The 0.3-kb *Bam*HI -*Sal*I (blunted) fragment from pLDLF007 was cloned into the *Bgl*II-*Sal*I (blunted) sites of pPR9tt to form pJLPM24::*lacZ*. The 0.3-kb *Bam*HI-*Sal*I fragment from pLDLF007 was cloned into the *Bgl*II-*Sal*I sites of pPR9tt to form pJLPM24fs::*lacZ*. The 5.1-kb *Sca*I (blunt) fragment from pJLPM24fs::*lacZ* was cloned into the *Eco*RV (blunt) site of pACYC184 to form pACPM24fs::*lacZ*. To construct pJLP::*lacZ*, the single *Bst*BI site of pPR9tt was disrupted by re-ligation of the pPR9tt *Bst*BI (blunted) fragment, resulting in pPR9tt-1. The 9.4-kb *Sal*I-*Hin*dIII fragment of pPR9tt-1 was ligated to a *Sal*I-*Hin*dIII DNA fragment amplified from pLDLF007 using LFR37 and JLU88 as primers. The 0.7-kb *Eco*RI-*Hin*dIII fragments of the PCR products generated by random PCR mutagenesis of *traM* (see below) were ligated to a 2.5-kb *Eco*RI-*Hin*dIII fragment of pRFM200, resulting in pRFM200 derivatives named

Table 2-1. Plasmids[a] and oligonucleotides

Plasmid & oligonucleotide	Description[f] & References
F_e*lac*	Tra^+Lac^+, transfer-repressed (Falkow and Baron, 1962)
F_e*lac*drd	Tra^+Lac^+, transfer-derepressed F_e*lac* derivative (Lu *et al.*, 2002)
JCFL0	Tra^+Lac^+, F'*lac* plasmid (Achtman *et al.*, 1972)
JCFL90	Tra^+Lac^+ $TraJ^-$, transfer-repressed F'*lac* plasmid (Thompson and Taylor, 1982)
pACYC177	Cloning vector; P15A replicon; Ap^RKm^R (Chang and Cohen, 1978)
pACYC184	Cloning vector; P15A replicon; Cm^RTc^R (Chang and Cohen, 1978)
pACPM24fs::*lacZ*	pACYC184 with a -1 frameshifted P_{traM}-*traM24-lacZ* fusion (This work)
pBAD24	Cloning vector; pMB1 replicon; Ap^R (Guzman *et al.*, 1995)
pBAD33	Cloning vector; P15A replicon; Cm^R (Guzman *et al.*, 1995)
pBADTraY	pBAD24 with F plasmid *traY* (Gubbins *et al.*, 2002)
pBF106	pBR322 with a 12-kb *Hin*dIII/*Xho*I fragment from pED208 (Finlay *et al.*, 1983)
pBF101	pACYC184 with a 28-kb *Hin*dIII fragment from pED208 (Finlay *et al.*, 1983)
pBF105	pBR322 with a 23.5-kb *Hin*dIII fragment from pED208 (Finlay *et al.*, 1983)
pBF111	pBR322 with a 7.5-kb *Xho*I fragment from pBF101 (Finlay *et al.*, 1983)
pBluescript KS+	Cloning vector; high-copy, pMB1-derived replicon; Amp^R (Short *et al.*, 1988)
pCR1[b]	Tra^- Mob^+, ColE1 derivative, $ColE1^R$ Km^R (Covey *et al.*, 1976)
pED208	Tra^+ Lac^+, transfer-derepressed F_e*lac* derivative (Finlay *et al.*, 1983)
pED104	pACYC177 with *finO* from the IncFII plasmid R6; Km^R (Lee *et al.*, 1992)
pGP1-2	pACYC184 with T7 RNA polymerase gene (Tabor and Richardson, 1985)
pJLac101	pPR9tt-1-derived transcriptional fusion-based promoter assessment plasmid (This work)
pJLac102	pJLac101 with F P_{traM} and *traM* upstream of the reporter gene (This work)
pJLac103	pJLac101 with F *traM* upstream of the reporter gene (This work)
pJLac104	pJLac101 with F P_{traM} upstream of the reporter gene (This work)

pJLac105	pJLac101 with P_{lac} from pBluescript KS+ upstream of the reporter gene (This work)
pJLac106	pJLac101 with F P_{traJ} (including *finP* and P_{finP} in the opposite direction) upstream of the reporter gene (This work)
pJLac107	pJLac101 with F P_{traJ} (including *finP* but no P_{finP} in the opposite direction) upstream of the reporter gene (This work)
pJLac108	pJLac101 with F P_{finP} upstream of the reporter gene (This work)
pJLac109	pJLac101 with F P_{traM}, *traM*, and T_{traM} upstream of the reporter gene (This work)
pJLac110	pJLac101 with F P_{traM}, *traM*, T_{traM}, P_{traJ} (including *finP* and P_{finP} in the opposite direction) upstream of the reporter gene (This work)
pJLac111	pJLac101 with F *oriT* and P_{traM} upstream of the reporter gene (This work)
pJLac112	pJLac101 with F *oriT*, P_{traM}, and *traM* upstream of the reporter gene (This work)
pJLac113	pJLac101 with an F fragment from *oriT* to P_{traY} upstream of the reporter gene (This work)
pJLac114	pJLac113 derivative with *traM* disrupted by a frameshift mutation (This work)
pJLac115	pJLac101 with F *oriT*, P_{traM}, *traM*, and T_{traM} upstream of the reporter gene (This work)
pJLac119	pJLac101 with F *oriT*, P_{traM}, *traM*, T_{traM}, and P_{traJ} (including *finP* and P_{finP} in the opposite direction) upstream of the reporter gene (This work)
pJLac121	pJLac101 with F *traM* and T_{traM} upstream of the reporter gene (This work)
pJLD331	pBAD33 with F *traD* (This work)
pJLED1	pBluescript KS+ with an 8-kb *Xho*I-*Hin*dIII fragment from pBF101 (This work)
pJLHD001	pT7-7 with a His_6-tagged *traD* (This work)
pJLJ001	pJLJF001 derivative without P_{finP} (This work)
pJLJF001	pT7-5 with an F *Dra*I-*Bgl*II fragment from P_{traJ} to P_{finP} (This work)
pJLM001	pT7-5 with F *traM* (This work)
pJLM002	pJLM001 derivative with a mutation, I109T, in *traM* (This work)
pJLM003	pRFM200 derivative with a mutation, I109T, in *traM* (This work)
pJLM004	pRFM200 derivative with a deletion of P_{finP} (This work)

pJLM004-MdeI	pJLM004 derivative with a deletion of most of *traM* (This work)
pJLM005	pT7-5 with F *traM* and T_{traM} (This work)
pJLM101	pT7-4 with P_{traM} and *traM* (This work)
pJLM102	pT7-4 with an F *Dra*I-*Bgl*II fragment from P_{traM} to P_{finP} (This work)
pJLM103	pJLM102 derivative with a *traM* mutation, *K31E* (This work)
pJLM104	pJLM102 derivative with a *traM* mutation, *I109T* (This work)
pJLM200	pT7-7 with a FLAG-tagged *traM* (This work)
pJLM201	pJLM200 derivative with a mutation, S79*, in *traM*
pJLM202	pJLM200 derivative with a mutation, K99E, in *traM*
pJLM203	pJLM200 derivative with a mutation, F121S, in *traM*
pJLM400	pBluescript KS+ with *traM* expressed from the *lac* promoter (This work)
pJLOM401	pBluescript KS+ with an F fragment from *oriT* to P_{finp} (This work)
pJLOM402	pBluescript KS+ with an F fragment from *oriT* to T_{traM} (This work)
pJLOM406	pBluescript KS+ with an F fragment from *oriT* to *traM* (This work)
pJLOY401	pBluescript KS+ with an F fragment from *oriT* to P_{traY} (This work)
pJLOY402	pJLOY401 derivative with *traM* disrupted by a frameshift mutation (This work)
pJLM501	P_{lac} and *traM* from pJLM4 replacing P_{lac} and the reporter gene in pJLac105 (This work)
pJLPM24::*lacZ*	pPR9tt-1 with an in-frame P_{traM}-*lacZ* fusion (This work)
pJLPM24::*lacZ*	pPR9tt-1 with an in-frame P_{traM} –*traM24-lacZ* fusion (This work)
pJLPM24fs::*lacZ*	pPR9tt-1 with a –1 frameshifted P_{traM}- *traM24-lacZ* fusion (This work)
pLDL100	pUC18 with pED208 *oriT* and *traM* gene (Di Laurenzio *et al.*, 1991)
pLDLF7	pUC18 with an F *Dra*I fragment containing P_{traM} and *traM* (Di Laurenzio *et al.*, 1992)
pLDLF007	pT7-4 with an F *Dra*I fragment containing P_{traM}, *traM* and T_{traM} (Di Laurenzio *et al.*,1992)
pNLK5	pBAD18 with F *traD* (Lee *et al.*, 1999)
pNY300	pUC18 with F *oriT* and *traM* (Frost *et al.*, 1989)

pOX38-Km	Tra$^+$ FinO$^-$, transfer-derepressed F derivative, Km^R (Chandler and Galas,1983)
pOX38-MK3	Tra$^+$ FinO$^-$ TraM$^-$, transfer-deficient F derivative, Km^R (Penfold *et al.*, 1996)
pOX38-Tc	Tra$^+$ FinO$^-$, transfer-derepressed F derivative, Tc^R (Anthony *et al.*, 1994)
pOX38-*traY244*	Tra$^+$ FinO$^-$ TraY$^-$, transfer-deficient F derivative, Km^R (Maneewannakul *et al.*, 1996)
pPR9tt^c	RK2 replicon; Amp^R Cm^R (Santos *et al.*, 2001)
pPR9tt-1	*Bst*BI site-disrupted pPR9tt derivative (This work)
pQE40	Cloning vector, Cm^R (Qiagen)
pRF105	pUC18 with R100 *oriT* and *traM* (Feket and Frost, 2000)
pRF400	pQE40 with F *traM* (Lu *et al.*, 2003)
pRF402	pRF400 derivative with a mutation, I109T, in *traM* (Lu *et al.*, 2003)
pRF911	pBR322-derived pBend2 with *sbmA* from the F plasmid (Fekete and Frost, 2002)
pRF940	pBR322-derived pBend2 with *sbmABC* from the F plasmid (Fekete and Frost, 2002)
pRFM200	pT7-5 with an F *Bst*BI-*Bgl*II fragment from *traM* to P_{finP} (Lu *et al.*, 2003)
pRFM200-Mdel	pRFM200 derivative with a deletion containing most of *traM* (This work)
pRS27	pSC101 with an F fragment from *oriT* to *traV* (Achtman *et al.*, 1978)
pRS29	pSC101 with an F fragment from *traV* to *traG* (Achtman *et al.*, 1978)
pRS31	pSC101 with an F fragment from *traG* to *FinO* (Achtman *et al.*, 1978)
pSC101	Cloning vector, Tet^R (Cohen and Chang, 1977)
pSnO104	pACYC184 with *finO* from the IncFII plasmid R6; Cm^R (Lee *et al.*, 1992)
pT7-4, pT7-5 & pT7-7	Cloning vector; pMB1 replicon; Amp^R (Tabor and Richardson, 1985)
pUC118	Cloning vector; high-copy; pMB1-derived replicon; Amp^R (Vieira and Messing, 1987)
pUC18	Cloning vector; high-copy, pMB1-derived replicon; Amp^R (Yanisch-Perron *et al.*, 1985)
R100	Tra$^+$, Cm^{R}Fa^{R}Sm^{R}Sp^{R}Su^{R}Tc^{R}Hg^R, transfer-repressed (Egawa and Hirota, 1962)
R100-1	Tra$^+$ FinO$^-$, Cm^{R}Fa^{R}Sm^{R}Sp^{R}Su^{R}Tc^{R}Hg^R, transfer-derepressed (Sugino and Hirota,

	1962)
R1162[d]	Tra$^+$ Mob$^+$, Sm^{R}Su^R ; IncQ plasmid (Barth and Grinter, 1974)
JLU2	5'GAATAAACGAAATTTGACTTCG3'; 5' end is the third nucleotide upstream of the *traM* stop codon, UAA
JLU3	5'CTATAGGGAGACCGGAATTCG3'; including the *Eco*RI site (underlined) in pT7-5
JLU4	5'CGATAAGCTTGGGCTGCAGG3', including the *Hin*dIII site (underlined) in pT7-5
JLU75	5'TAATAGATCTCACTATAGGGAGACCG3', complementing the 26 nucleotides immediately downstream of the *Eco*RI site in pT7-5 with 3 bases changed to produce a *Bgl*II site (underlined)
JLU76	5'ATAGGTACCATCGGATACATAGGAAC3', 5' complementing nucleotide 20 in F *traJ* with 4 bases changed to produce a *Kpn*I site (underlined)
JLU77	5'ATTAGAGATTACCTTTTTTGAAC3', 5' complementing nucleotide 21 in *traM*
JLU78	5'TTTGGATCCTTCATCATCATTTTTTGGAAAAAATCG3', between *gene X* and *oriT* in the F plasmid with 2 bases changed to produce an *Eco*RI site (underlined)
JLU80	5'TAGGCGTATCACGAGGCCC3', 5' beginning at nucleotide 4328 in pBR322
JLU81	5'GGTGCCTGACTGCGTTAGC3', 5' complementing nucleotide 64 in pBR322
JLU82	5'ATAGGTACCTTTGTGAGGAGGTTCC3', beginning at nucleotide 26 upstream of the *traJ* start codon with one base changed to produce a *Kpn*I site (underlined)
JLU88	5'GGTTCAAGCTTGCCATTAGAGATTACC3'; 5' complementing nucleotide 16 in *traM* with 4 bases changed to produce a *Hin*dIII site (underlined)
JLU94	5'ACGGGTACCAAATCTTTTCAATAACAC3', 5' complementing nucleotide 39 in F *traY* with 2 bases changed to produce a *Kpn*I site (underlined)
JLU95	5'GATACCAGGCGTTTCCC 3', beginning at nucleotide 1297 in pBluescript KS+
JLU96	5'GAAGATCTCCCAATACGCAAACCGCC3', 5' complementing 1036 in pBluescript KS+ with 4 bases changed to produce a *Bgl*II site (underlined).
JLU205	5'TAGAATTCGCgactacaaagacgacgatgacaagGCTAAGGTGAACCTGTATATC3', 5' starting from 2 nucleotides upstream of the *Eco*RI site (underlined) in pT7-7

	plus a FLAG tag (lower case) and the 21 nucleotides after the start codon in F *traM*
JLU207	5'TGGGGATCCTGAGAATTGAAGACTGGAG3', 5' complementing the 40th nucleotide downstream of F *traD* with 3 bases changed to produce a *Bam*HI site (underlined)
JLU208	5'TAGAATTCaccatcacacacaccatATGAGTTTTAACGCAAAGGATATG3', 5' starting from 2 nucleotides upstream of the *Eco*RI site (underlined) in pT7-7 plus a His6 tag (lower case) and the first 24 nucleotides of F *traD*
LFR37	5'AACAGCTATGACCATG3', 5' complementing nucleotide 823 in pBluescript KS+
LFR63	5'GGGTTTTCCCAGTCACGACG3', beginning at nucleotide 578 in pBluescript KS+
RFE6	5'GGATCCATGGCTAAGGTGAACCTG3'; a 5' *Bam*HI site (underlined) plus the first 18 nucleotides of *traM* beginning with the start codon
SPE8	5' CATAGGCATCATTGCTGATATACAG 3', 5' complementing nucleotide 40 in *traM*
SPE9	5'GAATTCTTATTCATCATCATTTTTTG3'; a 5' *Eco*RI site (underlined) plus 20-nucleotides complementing *traM* from its stop codon, UAA

[a] pRFM200 derivatives and pJLM400 derivatives, which contain mutated *traM*, are not listed here. These plasmids are named after the corresponding mutations as listed in Table 4-2.

[b] kindly provided by R. J. Meyer, School of Biology, University of Texas at Austin.

[c] kindly provided by S. Valla, Dept. of Biotechnology, Norwegian University of Science and Technology.

[d] kindly provided by D. E. Taylor, Department of Medical Microbiology and Immunology, University of Alberta.

[f] The abbreviations used are list in the "Abbreviations" section.

after the corresponding *traM* mutations (Table 4-2). The 0.7-kb *Eco*RI-*Bam*HI fragments containing wild type or mutated *traM* from pRFM200 or its derivatives were cloned into the *Eco*RI- *Bam*HI sites of pBluescript KS+, resulting in pJLM400 or pJLM400 derivatives, respectively. To reduce TraM expression from pJLM400 or its derivatives, glucose was added into the growth medium to a final concentration of 0.4% (w/v). A 0.7-kb *Bst*BI -*Kpn*I fragment from pRFM200, K31E, or I109T was used to replace the 0.5-kb *Bst*BI-*Kpn*I fragment in pLDLF007, resulting in pJLM102, pJLM103, or pJLM104, respectively. The 2.5-kb *Eco*RI-*Bam*HI fragment from pT7-7 was ligated to the 0.7-kb *Eco*RI-*Bam*HI fragments of DNA amplified from pRFM200, S79*, K99E, or F121S using JLU205 and JLU4 as primers, resulting in pJLM200, pJLFM201, pJLFM202, or pJLFM203, respectively. A 2.2-kb *Eco*RI-*Hin*dIII fragment from pNLK5 was cloned into the *Eco*RI-*Hin*dIII sites of pBAD33, resulting in pJLD331. The 2.5-kb *Eco*RI-*Bam*HI fragment from pT7-7 was ligated to a 2.2-kb *Eco*RI-*Bam*HI fragment of DNA amplified from pNLK5 using JLU208 and JLU207 as primers, resulting in pJLHD001.

The promoter assessment vector pPR9tt-1 was converted from measuring translational fusions to transcriptional fusions by constructing pJLac101. A 0.1-kb *Bst*BI (blunted)-*Sal*I (blunted) fragment from pLDLF007 was ligated to the 9.4-kb *Xho*I (blunted)-*Sal*I (blunted) fragment of pPR9tt-1 such that the RBS and first 24 codons of *traM* were fused in-frame to the 5'-truncated *lacZ* to give pJLac101. A 0.4-kb *Bst*BI -*Kpn*I fragment from pJLM001 was used to replace the 0.5-kb *Bst*BI-*Kpn*I fragment in pLDLF007, resulting in pJLM101. A 0.5-kb *Bst*BI -*Kpn*I fragment (*traM*) from pLDLF007 was used to replace the 0.7-kb *Bst*BI-*Kpn*I fragment in pRFM200, resulting in pJLM005. The 0.6-kb *Bam*HI-*Kpn*I fragment (P_{traM} plus *traM*) from pJLM101 was

cloned into the *Bgl*II-*Kpn*I sites of pJLac101 to give pJLac102. The *Bgl*II-*Kpn*I fragments of DNA amplified from pJLM001, pRFM200-Mdel, pJLM004-Mdel, or pJLM005 using the primers JLU75 and JLU4 were cloned into the *Bgl*II-*Kpn*I sites of pJLac101 to give pJLac103, pJLac106, pJLac107, or pJLac121, respectively. A 0.2-kb *Bam*HI-*Bst*BI (blunted) fragment (P_{traM}) from pLDLF007 was ligated to a 9.5-kb *Bgl*II-*Kpn*I (blunted) fragment of pJLac101 to give pJLac104. A *Bgl*II-*Kpn*I fragment (the *lac* promoter, P_{lac}, and the *lacO* operator) of DNA amplified from pBluescript KS+ using primers JLU96 and LFR63 was cloned into the *Bgl*II-*Kpn*I sites of pJLac101 to give pJLac105. A 0.8-kb *Bgl*II-*Xba*I fragment (P_{lac} plus *traM*) of DNA amplified from pJLM400 using primers JLU96 and LFR63 replaced the 3.1-kb *Bgl*II-*Xba*I fragment (P_{lac} plus *lacZ*) in pJLac105 to form pJLM501. The 0.4-kb and 0.6-kb *Bam*HI-*Kpn*I fragments from pLDLF007 and pJLM102 were cloned into the *Bgl*II-*Kpn*I sites of pJLac101 to form pJLac109 and pJLac110, respectively. A 0.1-kb *Kpn*I fragment of DNA amplified from pRFM200 using JLU4 and JLU82 as primers was cloned into the *Kpn*I site of pJLac101 such that P_{finP} expresses the reporter gene, resulting in pJLac108.

A 2.4-kp *Eco*RI (blunted)-*Kpn*I fragment of pRFM200 was ligated with a 0.2-kb *Dra*I-*Kpn*I fragment of pRFM200 or a 0.15-kb *Dra*I-*Kpn*I fragment of pJLM004, resulting in pJLJF001 or pJLJ001, respectively. A 2.9-kb *Eco*RI-*Kpn*I fragment of pBluescript KS+ was ligated to a 1.1-kb *Eco*RI-*Kpn*I fragment of DNA amplified from pNY300 using JLU78 and LFR37 as primers, resulting in pJLOM401. A 0.4-kb *Bst*BI-*Kpn*I fragment from pLDLF007 or a 0.4-kb *Bst*BI-*Kpn*I fragment from pJLM101 replaced the 0.65-kb *Bst*BI-*Kpn*I fragments in pJLOM401, resulting in pJLOM402 or pJLOM406, respectively. Religation of a blunted 3.7-kb *Sal*I-digested fragment, a 3.3-kb

*Sal*I (blunted)-*Eco*RV fragment, or a 3.2-kb *Eco*RI (blunted)-*Eco*RV fragment of pJLOM402, resulted in pJLOM403, pJLOM404, or pJLOM405, respectively. A 2.9-kb *Eco*RI-*Kpn*I fragment of pBluescript KS+ was ligated to an *Eco*RI-*Kpn*I fragment of DNA amplified from pRS27 using JLU78 and JLU94 as primers, resulting in pJLOY401. Religation of a blunted 4.5-kb *Sal*I-digested fragment of pJLOY401 resulted in pJLOY402. A 0.35-kb *Bam*HI-*Bst*BI (blunted) fragment (*oriT* plus P_{traM}) or a 1.1-kb *Bam*HI-*Kpn*I fragment from pJLOM401 was ligated to a 9.5-kb *Bgl*II-*Kpn*I (blunted) fragment of pJLac101 to give pJLac111 or pJLac119, respectively. A 9.5-kb *Bgl*II-*Kpn*I fragment of pJLac101 was ligated to a 0.85-kb *Bam*HI-*Kpn*I fragment from pJLOM402 or pJLOM406 or a 1.8-kb *Bam*HI-*Kpn*I fragment from pJLOY401 or pJLOY402, resulting in pJLac115, pJLac112, pJLac113, or pJLac114, respectively.

2.4 Random PCR mutagenesis of *traM*

Oligonucleotides JLU3 and JLU4 were used as primers for amplification of *traM* under error-prone PCR conditions (Chapter 4). Reaction mixtures contained: 5 ng of pRFM200, 7 µl of 10 mM dNTPs, 2 µl of 100 mM $MgSO_4$, 50 pmol of JLU3 and JLU4 each, 1 µl of *Taq* DNA polymerase (5 units/µl; Roche Diagnostics), 10 µl of 10 x *Taq* incubation buffer, and double distilled H_2O to make the total volume to 100 µl. The PCR reactions were performed in the following steps: 1) 95°C for 30 seconds; 2) 55°C for 30 seconds; 3) 72°C for 1 minute; 4) repeat steps 1, 2, and 3 for 40 cycles.

2.5 Selection of autoregulation-defective *traM* mutants

The detailed procedure for selecting autoregulation-defective *traM* mutants was described in Chapter 4. Briefly, potential *traM* mutants cloned in pRFM200 derivatives were transformed into DH5α cells containing pACPM24fs::*lacZ*. The transformed cells were plated on solid LB media containing X-gal, chloramphenicol, and ampicillin. Transformants were incubated for 24 hours at 37°C; and dark blue colonies were collected for further characterization. Plasmid DNA from each dark blue colony was extracted and sequenced using primers JLU3 and JLU4 to locate mutations in *traM*.

2.6 Selection of *traM* mutants normal for autoregulation but defective for F conjugation

The *traM* fragments generated by random PCR mutagenesis were cloned to form pRFM200 derivatives and transformed into DH5α cells carrying pOX38-MK3 and pACPM24fs::*lacZ*. The transformed cells were grown on LB plates with X-gal, ampicillin, chloramphenicol, and kanamycin at 37°C for 24 hours. Each light blue colony was patched on a Km-Amp plate and a Km-Spc plate covered with fresh ED24 cells. The light blue transformants that did not produce transconjugants on Km-Spc plates were selected for further characterization. Plasmid DNA was extracted and sequenced using primers JLU3 and JLU4 to locate mutations in *traM*.

2.7 β-galactosidase assays

A fresh, single colony was inoculated into LB broth containing appropriate antibiotics and grown at 37 °C with shaking for 3 hours (or 16 hours for cells containing pACPM24fs::*lac*). A 30-μl or 200-μl sample was used for determining β-galactosidase

activity as described by Miller (1972) and reported as Miller units (MU). The values were calculated using the equation: $1000(A_{420}/tvOD_{600})$, where t = time of reaction (minutes), and v = volume of culture added (ml).

2.8 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis

0.1 OD_{600} of exponentially growing cells (except where specified) were separated by a 15% SDS-polyacrylamide gel with a 7% stacking gel. The target protein was assayed by Coomassie blue staining (Sambrook *et al.*, 1989) or by immunoblot as described by Penfold *et al.* (1996). Rabbit anti-TraM (Di Laurenzio *et al.*, 1992), rabbit anti-TraD (Panicker *et al.*, 1992), rabbit anti-TraJ, rabbit anti-FinO, and rabbit anti-TraY antisera were used at a 1:10, 000 dilution, and monoclonal mouse anti-TraA antibodies (Anthony *et al.*, 1999) were used at 1:500 dilution.

2.9 Native and blue native polyacrylamide gel electrophoresis

Native polyacrylamide gel electrophoresis was performed as previously described (Speicher, 1995). A 15% continuous polyacrylamide gel (pH 8.8) with a 7% stacking gel (pH 6.8) was used and the gel was run at 4 °C and 20 mA for 16 hours. For blue native gel electrophoresis, a 6 to 15% gradient polyacrylamide gel (pH 7.0) with a 4% stacking gel (pH 7.0) was used. Blue native gel electrophoresis was performed as described by Schagger and von Jagow (1991) and Schagger *et al.* (1994). Proteins separated by both native gels and blue native gels were transferred to PVDF membranes for immunoblot analysis with anti-TraM antiserum. To prepare samples for native gels, DH5α cells containing pRFM200 or its derivatives were grown to mid-exponential phase. The pellet

from 5 OD_{600} of cells was re-suspended in 800 µl of sample buffer [62.5 mM Tris-HCl with one tablet of Complete, Mini protease inhibitor cocktail (Roche) per 10 ml of buffer; pH 6.8]. Cells were broken by sonication (10 seconds with a 15-second break, repeated 6 times, on ice) and centrifuged at 15,000 *g* and 4°C for 30 minutes. The supernatant was collected and stored at -80°C for later use.

2.10 Donor ability assays

E. coli XK1200 and ED24 were used as donor and recipient strains, respectively. Donor and recipient cells were grown to mid- and late- exponential phase in LB with appropriate antibiotics, respectively. Donors (50µl) and recipients (200 µl) were mixed in 1 ml of 37°C pre-warmed LB and incubated at 37°C for 30 minutes. Mating was terminated by vortexing vigorously and putting the cells immediately on ice to prevent further conjugation. After serial dilutions in cold SSC buffer (0.15M sodium chloride, 0.015M sodium citrate, pH 7.0), 10-µl portions of each dilution were spot-dropped onto selective plates containing combinations of antibiotics to select for donors and transconjugants. Plates were dried and incubated at 37°C overnight. Donor ability was calculated as the number of transconjugants divided by the number of donors.

2.11 Overexpression and purification of TraM and its mutants

BL21-DE3 cells containing pRFM200 or its derivatives were grown in 250 ml of LB containing ampicillin at 37°C with vigorous shaking. After 3 hours, IPTG was added to the culture to a final concentration of 1mM and the culture was grown for another 2 hours before harvesting. Approximately 150 OD_{600} of cells were used for purification of each

protein. The cell pellet was suspended in 6 ml of B-Per® bacterial protein extraction reagent (Pierce) with one tablet of Complete, Mini protease inhibitor cocktail (Roche); and the soluble fraction of the cells was extracted according to the manufacturer's instructions. All the following steps were performed at 4°C or on ice. Ammonium sulfate (720 mg) was dissolved in the extracted soluble fraction. After centrifugation at 27,000 x *g* for 10 minutes, the supernatant was transferred into a new centrifuge tube, in which 480 mg of ammonium sulfate was dissolved. After centrifugation at 27,000 x *g* for 10 minutes, the supernatant was carefully aspirated and discarded. The precipitate was dissolved in 2 ml of malonic acid (50 mM, pH 5.5), and the solution was centrifuged at 27,000 x *g* for 10 minutes. The supernatant was brought to 2.5 ml with malonic acid (50 mM, pH 5.5), and was desalted on a PD10 column (Amersham). After passing through a 0.45-μM Millex® syringe driven filter (Millipore), the desalted protein extract was loaded onto a cation-exchange column (MonoS HR 5/5, Amersham) using an Amersham FPLC model LCC-500. The column was eluted with malonic acid (50 mM, pH 5.5) and a 0 to 1 M NaCl gradient. Because TraM has very low UV absorbance, eluted fractions were examined by 15% SDS-polyacrylamide gels with Coomassie blue staining, and protein peaks were further confirmed by immunoblot with anti-TraM antisera. TraM, V4A, A37V, R48C, Q53L, Q78H, K99E, V106A, and F120L were eluted at 0.5 to 0.7 M NaCl. N5D, N10D, and S79* were eluted at 0.3 to 0.5 M NaCl. I109T, F120S, F121V, and F121S were eluted at 0.45 to 0.65 M NaCl. The pooled MonoS fractions (3-5 ml) of TraM or its mutant proteins were loaded onto a size exclusion column (Hiload® 16/60 Superdex 75 prep grade, Amersham). The column was eluted with SEC buffer (50mM sodium phosphate, 150 mM NaCl, pH 7.2), and the eluate was collected in 2-ml fractions.

Fractions from B9 (void volume) to E12 (one column volume) were examined. The major peak fractions of each protein were concentrated, desalted, and the buffer was exchanged for Tris-HCl (50mM; pH 7.6) using an Amicon® ultracentrifuge filter (Millipore) to a final volume of 50 µl. Protein concentration was determined using BCA protein assays (Pierce) following the manufacturer's instructions.

2.12 Analytical size exclusion chromatography (Analytical SEC)

Purified TraM or its mutants (5 µg) was brought to 1 ml with SEC buffer and loaded onto a Hiload® 16/60 Superdex 75 prep grade column at 4°C in FPLC. The column was eluted at 0.5 ml/minute with 120 ml (one column volume) of SEC buffer, and the eluate was collected in 2-ml fractions. Samples (10 µl) from different fractions were separated on a 15% SDS-polyacrylamide gel and analyzed by immunoblot with anti-TraM antiserum. The column was calibrated with molecular weight markers (Sigma) under the same chromatographic conditions. The column was calibrated with molecular weight markers (Sigma) under the same chromatographic conditions.

2.13 Electrophoretic mobility shift assays (EMSA)

DNA fragments containing *sbmA* and *sbmABC* were amplified by PCR from pRF911 and pRF940, respectively, using primers JLU80 and JLU81. The resulting mixtures were concentrated in a Savant SpeedVac and separated by a 2% agarose gel. The *sbmA* and sbm*ABC* fragments were isolated from the agarose gel and were quantified using an Ultrospec 3000 (Amersham). Each binding reaction contained 40 nM of *sbmA* or *sbmABC*, 50 mM Tris-HCl (pH 7.6), 10% glycerol, 1 mM dithiothreitol, 30 µg/ml bovine

serum albumin, and 1.5 µg of poly(dI·dC) with a final volume of 15 µl. After addition of a specified amount of purified proteins, reaction mixtures were incubated at 30°C for 20 minutes. The resulting mixture was added with 3 µl of 6x load dye (0.25% bromophenol blue, 30% glycerol) and loaded onto a 2% agarose gel that had been pre-run at 4°C and 30 mA in TBE (90 mM Tris-borate, 1 mM EDTA) for 30 minutes. The loaded gel was run at 4°C and 30 mA until the bromophenol blue dye reached the bottom of the gel. DNA was visualized by ethidium bromide staining.

2.14 Overexpression and solubilization of His_6-TraD

BL21-DE3 cells containing pJLHD were grown in 500 ml of LB broth containing ampicillin at 37°C with vigorous shaking. After 3 hours, IPTG was added to the culture to a final concentration of 1 mM and the culture was grown for another 2 hours before harvesting. His_6-TraD was dissolved followed previously described procedures (Panicker *et al.*, 1992). Approximately 250 OD_{600} of cells were pelleted and suspended in 10 ml buffer A [50 mM Tris-HCl (pH 7.8), 0.4 mg/ml lysozyme (Sigma)] plus one tablet of Complete, Mini protease inhibitor cocktail (Roche). The suspension was incubated at 37°C for 30 minutes when the suspension was viscous. DNase (50 units; Roche) and 150 µl of $MgCl_2$ (1M) were added to the suspension and incubated at 37°C for 15 minutes until the suspension was no longer viscous. The suspension was then lysed by sonication on ice for 3 minutes (30 seconds with a 30-second break, repeated 6 times) at maximum output. The unlysed cells were removed by a low-speed centrifugation (SS34 rotor, 5,500 rpm, 4°C, 15 minutes) and the membrane fraction was collected by a high-speed centrifugation (SS34 rotor, 15,000 rpm., 4°C, 30 minutes). The collected membrane

fraction pellet was resuspended in 7.5 ml of buffer B [50 mM Tris-HCl (pH 7.8), 10 mM imidazole, 10% glycerol, 1.5% Triton X-100, 300 mM NaCl] plus one tablet of Complete, Mini protease inhibitor cocktail. The suspension was incubated at 4°C for 5 hours with gentle shaking and was centrifuged at medium speed (SS34 rotor, 8,000 rpm, 4°C, 15 minutes) to clear the undissolved membrane fraction. The supernatant, which contained solubilized TraD, was used immediately for affinity chromatography or stored at -80°C for later use.

2.15 Analysis of TraM-TraD interactions using affinity chromatography

The procedure mainly followed the manufacturer's instructions (Qiagen). Solubilized TraD extract (1.5 ml) was mixed with 20 µl of 50% Ni-NTA resin (Qiagen) gently at 4°C overnight. The resin was pelleted by centrifugation at 15,000 x *g* for 10 seconds and was washed for two times with 100 µl of cold buffer C [50 mM Tris-HCl (pH 7.8), 20 mM imidazole, 1% Triton X-100, 300 mM NaCl]. The washed resin was suspended in 1 ml of buffer D [50 mM Tris-HCl (pH 7.8), 10 mM imidazole, 1% Triton X-100, 150 mM NaCl] plus one fifth tablet of Complete, Mini protease inhibitor cocktail (Qiagen). BSA (40 µg) and 0.3 µg of purified TraM (or one of it mutant proteins) were added to the suspension and mixed gently at 4°C for 5 hours. The resin was pelleted by centrifugation at 15,000 x *g* for 10 seconds and washed with 100 µl of cold buffer C for three times. The washed resin was eluted 3 times with 20 µl of elution buffer [50 mM Tris-HCl (pH 7.8), 250 mM imidazole, 1% Triton X-100, 300 mM NaCl]. A 2 µl volume out of the pooled 60-µl eluate was run on a 15% SDS-polyacrylamide gel and assayed by immunoblot with anti-TraM antisera or anti-TraD antisera. His_6-tagged TraK (Manchak and Frost,

unpublished data) was used as a negative control for His_6-tagged TraD interactions with TraM.

2.16 Analysis of TraM-TraD interactions using co-immunoprecipitation assays

The procedure mainly followed manufacturer's instructions (Sigma). BL21-DE3 cells containing pJLD331 and a plasmid expressing FLAG-tagged TraM (or one of its mutant proteins) were grown in 10 ml of LB containing chloramphenicol and ampicillin at 37°C with vigorous shaking. After 2 hours, arabinose was added to the culture to a final concentration of 0.1% and the culture was grown for another 3 hours before harvesting. Approximately 5 OD_{600} of cells were pelleted and suspended in 1 ml of IP buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 10% glycerol] plus one fifth tablet of Complete, Mini protease inhibitor cocktail (Qiagen). Lysozyme (10 μg) was added and mixed at 4°C for 30 minutes. The suspension was lysed by sonication on ice for 1 minute (10 seconds with a 15-second break, repeated 6 times) at a medium output. The lysate was centrifuged at 15,000 x *g* and 4°C for 15 minutes, and the supernatant was transferred into a tube containing 20 μl of 50% anti-FLAG M2 agarose gel (Sigma). The mixture was incubated at 4°C for 3 hours with gentle shaking. The resin was pelleted by centrifugation at 10,600 x *g* for 5 seconds and washed for 3 times with 0.5 ml of TBS buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl]. The washed resin was mixed with 100 μl of glycine-HCl (0.1 M; pH 3.5) and incubated for 5 minutes at room temperature. The mixture was centrifuged at 10,600 x *g* for 5 seconds, and the supernatant was transferred into a fresh tube containing 10 μl of 10 x TBS buffer. Either 2 μl or 10 μl of supernatant was separated by 15% SDS-

polyacrylamide gel and assayed by immunoblot with anti-TraM or anti-TraD antisera, respectively.

2.17 Viable cell and ampicillin-resistant cell count

Serial 10^{-1} dilutions of cells containing different plasmids were prepared in cold SSC buffer. Each dilution (100 µl) was plated in duplicate onto both LB plates and LB plates containing ampicillin. After 24 hours of incubation at 37°C, plates containing between 30 and 300 colonies (or CFU, colony forming units) were used for calculating viable cells with the equation: CFU x 10^{n+1} cells/ml, where n= number of dilutions.

2.18 Determining growth curves

Fresh transformant colonies with different plasmids were inoculated into 1.5 ml of LB broth and grown for 12 hours at 37°C with or without 0.8% glucose to control the P_{lac} promoter. The 12-hour cultures were diluted into fresh media to give an OD_{600} of approximately 0.06. Cells were grown at 37°C with vigorous shaking and the OD_{600} and viable cell count were measured at 60-minute intervals. OD_{600} or the logarithm of CFU per milliliter of culture was plotted against time. Each sample was measured in duplicate; and each experiment was repeated once with no significant deviations observed.

2.19 Epidemic spread assays

Approximately 4.5 x 10^7 *E.coli* XK1200 cells containing pSnO104 and pOX38Km were mixed with 3 x 10^8 *E.coli* XK1200 cells containing pSnO104 and pT74 (or pLDLF007) in 1-ml LB broth containing chloramphenicol at 37°C without shaking for 18

hours. Total viable cell count and pOX38-Km-containing viable cell count were performed on Cm and Km-Cm plates, respectively. The transfer efficiency of pOX38-Km in the 18-hour mating mixture was assayed by adding 50 µl of the 18-hour mating mixture into 1ml pre-warmed LB broth together with 200µl late-exponential culture of *E.coli* ED24 cells following procedures of a standard donor ability assay.

Chapter 3

Role of *traM* in *tra* Gene Regulation

3.1 Introduction

Conjugation is one of the major mechanisms by which bacteria acquire new genes to exploit new environments and respond to selective pressure. Conjugation-related genes are normally not essential for bacterial growth, and expression of these genes increases the sensitivity of host cells to bacteriophages. Therefore, various mechanisms have evolved to regulate conjugation-related genes in different transfer systems (Zechner *et al.*, 2000).

The F plasmid is the paradigm for a large group of conjugative plasmids in the IncF incompatibility complex that carry genes important for human and veterinary medicine, such as antibiotic resistance and toxin production (Ippen-Ihler and Skurray, 1993). Conjugation-related genes or sequences in F-like plasmids are concentrated in the transfer (*tra*) region, in which *oriT* (origin of transfer), *traM*, *traJ*, and the major *tra* operon align in order (Figure 1-1; Frost *et al.*, 1994). The major *tra* operon that contains most of the conjugation-related genes is repressed by the FinOP fertility inhibition system in F-like plasmids with only a few known exceptions (Finnegan and Willetts, 1972; Gasson and Willetts, 1975; Chapter 8). FinP, an antisense RNA that binds to the 5' untranslated region of *traJ* mRNA, prevents translation of TraJ, an essential positive regulator for transcription of the major *tra* operon (Cuozzo and Silverman, 1986; van Biesen and Frost, 1994; Koraimann *et al.*, 1996). The FinO protein prevents degradation of FinP by ribonuclease E and acts as a chaperone to facilitate FinP-*traJ* mRNA interactions (Lee *et al.*, 1992; Jerome *et al.*, 1999; Arthur *et al.*, 2003). Therefore, most F-like plasmids transfer at very low frequency, whereas the F plasmid transfers at more than 100 fold-higher frequency due to disruption of *finO* by an IS*3* element (Cheah and

Skurray, 1986; Yoshioka *et al*, 1987). As FinO proteins encoded by different F-like plasmids are nearly identical and highly cross-reactive, supplying foreign FinO *in trans* can restore FinOP inhibition of the F plasmid (van Biesen and Frost, 1994; Frost *et al.*, 1994). The transfer ability of the F plasmid and levels of most *tra* gene products are maximal during early exponential phase and decrease to undetectable levels in stationary phase (Frost and Manchak, 1998), suggesting that mechanisms other than FinOP inhibition also regulate *tra* gene expression.

F plasmid *traM* has two promoters that are repressed by TraM binding to sites overlapping the transcriptional start sites (Di Laurenzio *et al.*, 1992; Penfold *et al.*, 1996). Anecdotal evidence of TraM toxicity begs the question why the strong *traM* promoters in the F plasmid have been maintained throughout evolution. In R1 and R100, *traM* has been suggested to positively regulate the major *tra* operon by transcriptional readthrough into *traJ* to upregulate TraJ expression (Polzleitner *et al.*, 1997; Dempsey, 1994; Stockwell and Dempsey, 1997). In the F plasmid, a transcript that hybridizes to a *traJ* probe corresponds in size to a *traM-traJ* dicistronic mRNA (Lee *et al.*, 1992), suggesting existence of transcriptional readthrough from *traM* into *traJ*. A previous study has shown that TraY (encoded by the major *tra* operon) positively regulates *traM* transcription (Penfold *et al.*, 1996), suggesting that a regulatory circuit might exist in the F plasmid to balance expression of all *tra* genes.

Some naturally transfer-repressed plasmids such as ColI factors have de-repressed transfer ability for 2 to7 generations in new transconjugants (Stocker *et al.*, 1963; Monk and Clowes, 1964). This phenomenon is called "high frequency of transfer" or "epidemic (or infectious) spread", which ensures plasmid dissemination throughout a recipient cell

population. Transcriptional readthrough from *traM* into *traJ* has been proposed to play a role in the possible epidemic spread of R100, a transfer-repressed IncFII plasmid (Dempsey, 1994); however, the mechanism of epidemic spread has not been investigated in detail.

In this work, the strength of different promoters in the F *tra* region and the levels of *traM* autoregulation and transcriptional readthrough were determined using a low-copy promoter assessment plasmid. Potential toxicity of TraM as well as host adaptations to high levels of TraM was investigated. The importance of P_{traM} for F conjugation and epidemic spread of transfer-repressed F-like plasmids were further studied. TraY-dependent *traM* expression was also characterized. The results suggest that *traM* negatively regulates its own expression to avoid potential TraM toxicity and positively regulates other *tra* genes by increasing *traJ* transcription via transcriptional readthrough, whereas TraY de-represses an *in cis* inhibition mechanism that coordinates expression of *traM* and the major *tra* operon.

3.2 Results

3.2.1 Promoter activity of different fragments in the F *tra* region

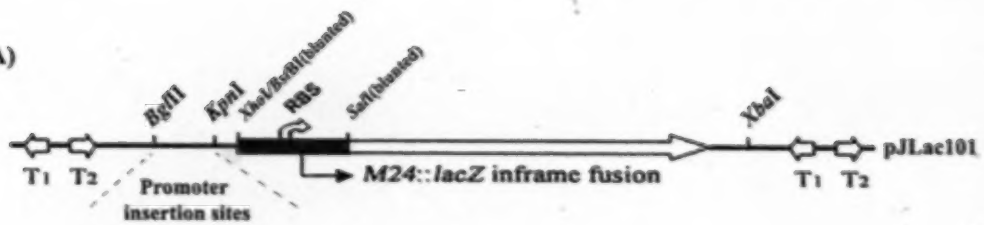
To understand the activity of gene transcription in the F *tra* region, I constructed a low-copy promoter assessment plasmid (RK2 replicon), pJLac101 (Figure 3-1A), which is capable of measuring promoters inserted as transcriptional fusions. The activity of the *lac* promoter (P_{lac}) was also determined to serve as a control (Figure 3-1A).

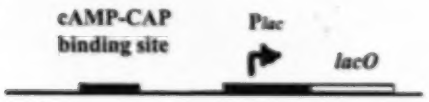
Promoters for *traM*, *traJ*, *finP*, and the major *tra* operon (P_{traM}, P_{traJ}, P_{finP}, and P_{traY}, respectively) in the F *tra* region have been annotated by Frost *et al.* (1994; Figure 3-1B). The strength of P_{traM} determined in the presence or absence of *traM* was 640 MU (pJLac102) or 7530 MU (pJLac104), respectively. The presence of *oriT* and the TraY binding sites did not affect the strength of P_{traM} significantly in the presence of TraY supplied by pOX38-MK3 (pJLac111 and pJLac112), indicating that TraY did not directly increase the strength of P_{traM}. The transcriptional activity within the coding region of *traM* was at 440 MU (pJLac103), indicating the presence of internal promoters (P_{int}) in *traM*.

The strength of P_{lac} in the presence of IPTG (pJLac105; 3640 MU) was about half that of P_{traM} and decreased to 648 MU in the presence of 0.4% glucose. P_{finP} (pJLac108) had an activity of 1960 MU. The strength of P_{traJ} with P_{finP} in the opposite direction (pJLac106) was 2200 MU, whereas the strength of P_{traJ} without P_{finP} (pJLac107) was 5450 MU, suggesting that transcription of *finP* counteracted *traJ* transcription. The strength of P_{traY} was 1380 MU in the presence of TraJ but decreased by more than 7-fold in the presence of R100 FinO supplied *in trans* (pJLac113; 180 MU).

Figure 3-1. Promoter strength of different fragments determined using pJLac101. The names of the corresponding constructs and the promoter strength of the fragments were indicated on the right of the figure. (A) The promoter assessment plasmid, pJLac101 and the strength of the *lac* promoter, P_{lac} (pJLac105). Glucose was used at a final concentration of 0.4% (w/v). IPTG was used at a final concentration of 1mM. Unique restriction sites (including promoter insertion sites) in pJLac101 are indicated above the figure. The black box in pJLac101 represents the first 24 codons of F *traM* plus the RBS that fused the *lacZ* reporter gene to form *M24::lacZ*. T1 and T2 in pJLac101 are terminators flanking the cloning sites and reporter gene. Arrows indicate the direction of promoters, genes, terminators, or ribosome binding sites. The terminal restriction sites of each fragment are indicated. *sbmA* and *sbmB* are TraM binding sites overlapping P_{traM} (P_{M1} and P_{M2}). CAP stands for Catabolite Activator Protein. (B) Promoter strength of fragments in the F *tra* region. The F *tra* region from *oriT* to the beginning of *traY* is illustrated at the top of the figure. The lines below represent different fragments corresponding to certain *tra* regions that are inserted in pJLac101 for assessing promoter strength. Promoter strength is represented by β-galactosidase activity in Miller units (MU). Black boxes represent genes, protein-binding sites, and sequence motifs. The open box represents incomplete *traY* with an arrow indicating its orientation. Angled arrows indicate the location and the direction of promoters. P_{m1} and P_{m2} are the two *traM* promoters (collectively called P_{traM}). P_{int} is the internal *traM* promoter(s). *sbmA*, *B*, and *C* are the three TraM binding sites. *sbyA*, *B*, and *C* are the three TraY binding sites. Restriction sites are shown under the lower strand. Other abbreviations used are list in the "Abbreviations" section.

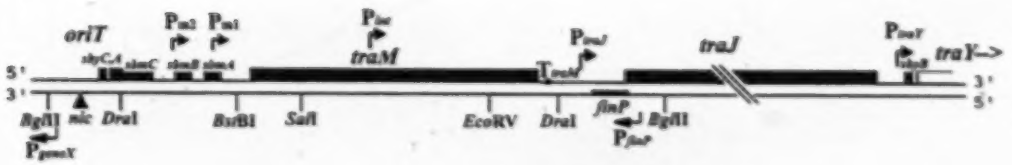
(A)





pJLac105 (650 +/- 90 MU; glucose-repressed)
(3640 +/- 100 MU; IPTG-induced)

(B)



pJLac102 (640 +/- 20 MU)
pJLac112 (700 +/- 35 MU, pOX38-MK3 present)
pJLac103 (440 +/- 30 MU)
pJLac104 (7530 +/- 400 MU)
pJLac111 (7720 +/- 550 MU, pOX38-MK3 present)

pJLac110 (2560 +/- 430 MU)
pJLac119 (2670 +/- 300 MU,
pOX38-MK3 present)

pJLac106 (2200 +/- 320 MU)
pJLac107 (5450 +/- 390 MU)
pJLac108 (1960 +/- 180 MU)

pJLac113
Without FinO : (1380 +/- 160 MU)
With R100 supplying FinO: (180 +/- 20 MU)

pJLac121 (280 +/- 10 MU)
pJLac109 (420 +/- 30 MU)
pJLac115 (440 +/- 20 MU,
pOX38-MK3 present)

There is a predicted ρ-independent transcriptional terminator for *traM* (T_{traM}) immediately upstream of the *traJ* promoter region (Frost *et al.*, 1994). However, approx. two-thirds of the activity of P_{int} (pJLac121) or repressed P_{traM} plus P_{int} (pJLac109) were detected downstream of T_{traM} (compare pJLac121 to pJLac103, or pJLac109 to pJLac102, respectively), indicating existence of transcriptional readthrough from *traM* into *traJ*.

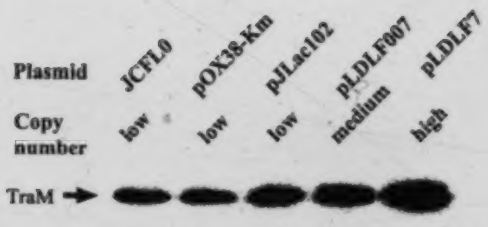
3.2.2 Autoregulation of *traM* avoids potentially toxic effects of TraM overexpression

F plasmid *traM* is stringently autoregulated as shown by the promoter analysis (Figure 3-1A), implying that unregulated TraM expression is deleterious. pLDLF7, pLDLF007, JCFL0, pOX38-Km, and pJLac102 each expresses *traM* from P_{traM}, but their copy numbers per chromosome vary from 1-2 (JCFL0 and pOX38-Km) to hundreds (pLDLF7). The level of TraM expressed by pLDLF7 was disproportionately low considering that pLDLF7 has up to over a hundred fold higher copy number than the other three plasmids (Figure 3-2A). This further indicated that the level of TraM is independent of the copy number of *traM*, a trait of the autoregulatory gene.

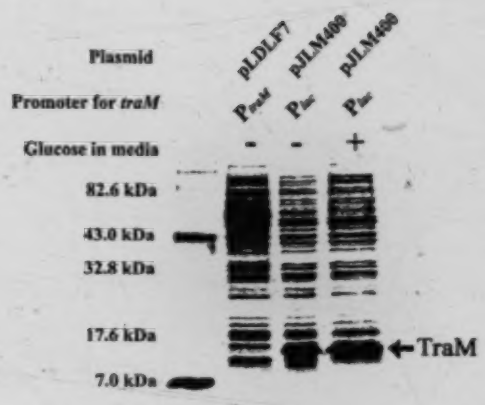
pLDLF7 did not express TraM that was detectable by Coomassie blue staining on a polyacrylamide gel, whereas pJLM400, which has the same copy number as pLDLF7 but expresses *traM* from P_{lac} instead of P_{traM}, did (Figure 3-2B). Cells containing pJLM400 formed tiny colonies on LB plates without 0.8% glucose (data not shown). OD_{600} of cells containing pJLM400 increased more slowly and reached lower maximal levels than that of the cells containing pBluescript KS+; and cells containing pJLM400 in media without 0.8% glucose reached a maximum OD_{600} value below one (Figure 3-2C). Cells containing pJLM400 in the absence of glucose were approximately 3-5 times longer than

Figure 3-2. Effects of TraM overexpression on cell growth. (A) Levels of TraM expressed from P_{traM} in plasmids with different copy numbers. TraM was detected by immunoblot analysis with TraM antiserum. Plasmid copy number is indicated according to the replicon of each plasmid (Table 2-1). Low: JCFL0 and pOX38-Km (1-2 copies per chromosome; Willetts and Skurray, 1987); pJLac102 (5-8 copies per chromosome; pogliano *et al.*, 2001). Medium: pLDLF007 (10 to 50 copies per chromosome; Sambrook *et al.*, 1989). High: pLDLF7 and pJLM400 (> 200 copies per chromosome; Sambrook *et al.*, 1989). (B) Levels of TraM expressed by different high-copy plasmids. TraM was detected by Coomassie blue staining on a 15% SDS-polyacrylamide gel. (C) OD_{600} versus time of cells expressing TraM at low or high levels. pBS KS+ (G-), cells containing pBluescript KS+ grown in the absence of glucose. pJLM400 (G+), cells containing pJLM400 in the presence of 0.8% glucose. pJLM400 (G-), cells containing pJLM400 in the absence of glucose. (D) CFU/ml versus time of cells expressing TraM at low or high levels. CFU, colony forming units.

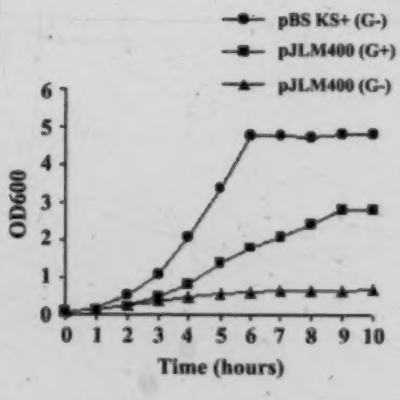
(A)


Plasmid
JCFL0
pOX38-Km
pJLac102
pLDLF007
pLDLF7
Copy number
low
low
low
medium
high
TraM

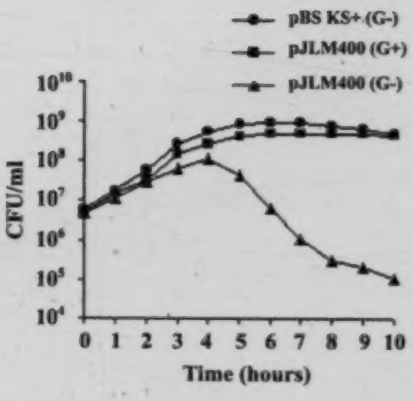
(B)


Plasmid
pLDLF7
pJLM400
pJLM400
Promoter for traM
P_{traM}
P_{tac}
P_{tac}
Glucose in media
-
-
+
82.6 kDa
43.0 kDa
32.8 kDa
17.6 kDa
7.0 kDa
TraM

(C)


pBS KS+ (G-)
pJLM400 (G+)
pJLM400 (G-)
OD600
Time (hours)

(D)

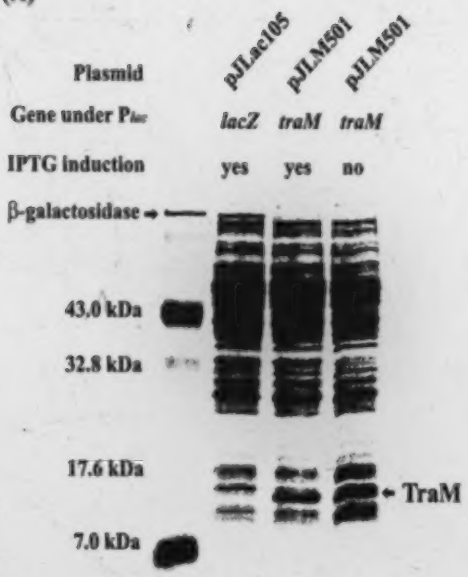

pBS KS+ (G-)
pJLM400 (G+)
pJLM400 (G-)
CFU/ml
Time (hours)

the cells containing pBluescript KS+ as measured under a transmission electronmicroscope (data not shown), suggesting defective cell division caused by TraM overexpression. The number of viable cells containing pJLM400 increased more slowly than that of the cells containing pBluescript KS+ (Figure 3-2D). In particular, the number of viable cells containing pJLM400 decreased dramatically after 4 hours of growth in medium without 0.8% glucose. Thus, high levels of TraM repressed cell growth and extremely high levels of TraM, which were expressed by pJLM400 when P_{lac} was not repressed by glucose, caused cell death.

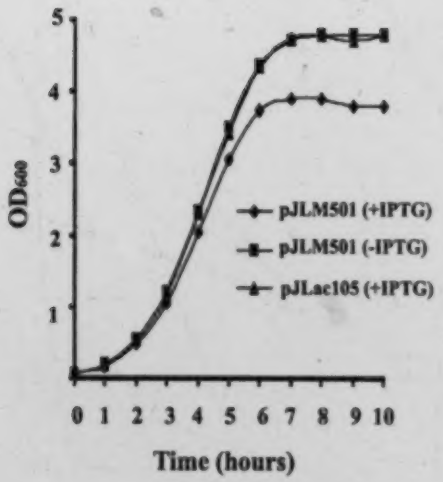
Mutating the TraM binding sites to abolish autoregulation could alter P_{traM} consequently; thus I did not study the effects of TraM expression from an unregulated P_{traM} in the F plasmid. Instead, I constructed pJLM501 based on the RK2 replicon, which is comparable to the F plasmid in copy number but expresses *traM* from P_{lac}, to mimic TraM overexpression from the unregulated P_{traM} in the F plasmid. When P_{lac} was induced by IPTG, cells containing pJLM501 expressed TraM at levels detectable by Coomassie blue staining and grew more slowly than cells containing pJLac105, which is identical to pJLM501 except that *lacZ* instead of *traM* is expressed from P_{lac} (Figure 3-3A and B). When P_{lac} was induced, conjugative transfer of pOX38-MK3 (a *traM*-deficient F derivative) was restored by pJLM501 to an approx. ten-fold lower level (0.07 transconjugants/donor) than when P_{lac} was not induced (0.6 transconjugants /donor). These results indicated the potential for the strong *traM* promoters in the F plasmid to affect cell growth rate and donor ability and underscored the need for the tight regulation of these promoters.

Figure 3-3. Overexpression of TraM and β-galactosidase by low copy number plasmids. (A) Levels of TraM and β-galactosidase. TraM and β-galactosidase were detected by Coomassie blue staining of a 15% SDS-polyacrylamide gel. (B) Growth curves of cells overexpressing TraM or β-galactosidase from low copy number plasmids. pJLM501 (IPTG), cells containing pJLM501 grown in the presence of 1mM IPTG. pJLM501, cells containing pJLM501 in the absence of IPTG. pJLac105 (IPTG), cells containing pJLac105 in the presence of 1mM IPTG.

(A)


Plasmid
pJLac105
pJLM501
pJLM501
Gene under Ptac
lacZ
traM
traM
IPTG induction
yes
yes
no
β-galactosidase
43.0 kDa
32.8 kDa
17.6 kDa
7.0 kDa
TraM

(B)


OD600
5
4
3
2
1
0 1 2 3 4 5 6 7 8 9 10
Time (hours)
pJLM501 (+IPTG)
pJLM501 (-IPTG)
pJLac105 (+IPTG)

3.2.3 Host cells can adapt to avoid toxic levels of TraM

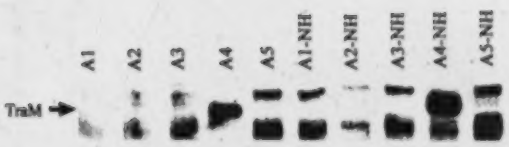
Occasionally, a large (normal size) colony of DH5α cells transformed with pJLM400 was found on LB plates without 0.8 % glucose (data not shown). Five such large colonies were collected for further study. Viable cell counts on solid media with or without ampicillin gave identical numbers, indicating that the cells maintained the plasmid (carrying ampicillin resistance) without experiencing cytotoxicity. Re-streaking of these large colonies on LB plates without 0.8% glucose continued to give large colonies, suggesting that the cells had adapted in some way.

These five apparently "adapted" strains were named A1 to A5. A1, A2, A3, and A5 did not express TraM detectable by Coomassie blue staining on SDS-polyacrylamide gels whereas A4 expressed lowered levels of TraM (Figure 3-4A). Plasmids extracted from A1 to A5 were transformed into plasmid-free DH5α cells to give A1-NH to A5-NH, respectively where NH represents "new host". Without 0.8% glucose in media, A4-NH formed tiny colonies and produced high levels of TraM whereas A1-NH, A2-NH, A3-NH, and A5-NH continued to form large colonies and did not express detectable TraM. This suggested that A1, A2, A3 and A5 resulted from changes to *traM* expression in pJLM400 whereas A4 resulted from mutations occurred in the original host chromosome.

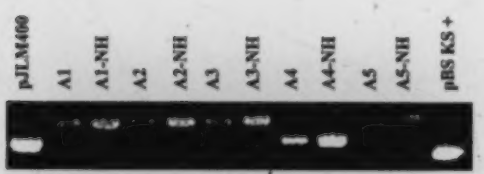
In order to characterize changes in the plasmids in adapted strains, plasmid DNA from equivalent numbers of cells was extracted and resolved by 1% agarose gels (Figure 3-4B). Strains A1 to A5 all contained less plasmid DNA than A1-NH to A5-NH, indicating that the copy number of the plasmids in A1-A5 was lowered due to host mutations. In addition, the plasmids from A1, A2, A3, and A5 were larger than their

Figure 3-4. Host adaptations to high levels of TraM. (A) Levels of TraM in adapted and new hosts. TraM was detected by Coomassie blue staining of a 15% SDS-polyacrylamide gel. Each lane was loaded with 0.1 OD_{600} of A1 to A5 or A1-NH to A5-NH cells grown in LB without glucose supplementation. (B) Levels of plasmid DNA in adapted and new hosts. Five microliters of DNA extracted from 3 OD_{600} cells (50 µl total) was loaded on a 1% agarose gel visualized by ethidium bromide staining. (C) Location of IS*5* insertion and sequencing primers in pJLM400. Some of the unique restriction sites in pBluescript KS+ are indicated. The RBS and the coding region of *traM* are indicated with black boxes. An angled arrow indicates the direction and location of the *lac* promoter. *oriV* refers to the ColE1 replicative origin. Arrows below the line indicate location and direction of primers for sequencing *traM* and the origin of replication in pJLM400 and its derivatives. The white triangle indicates the location of IS*5* insertion in pJLM400 in the adapted strains. Sequences adjacent to the insertion site are shown below. The RBS and start codon for *traM* is underlined; the italicized nucleotides refer to the direct repeats on either side of IS*5*; the arrow under IS*5* indicates the orientation of the IS*5* transposase gene.

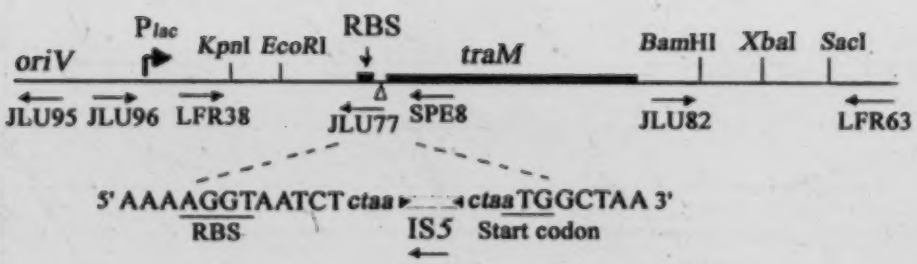
(A)


A1
A2
A3
A4
A5
A1-NH
A2-NH
A3-NH
A4-NH
A5-NH
TraM

(B)


pJLM400
A1
A1-NH
A2
A2-NH
A3
A3-NH
A4
A4-NH
A5
A5-NH
pBS KS +

(C)


oriV
Ptac
KpnI
EcoRI
RBS
traM
BamHI
XbaI
SacI
JLU95
JLU96
LFR38
JLU77
SPE8
JLU82
LFR63
5' AAAAGGTAATCT ctaa
ctaa TGGCTAA 3'
RBS
IS5
Start codon

parental plasmid (pJLM400), whereas A4 remained the same size and had the same copy number in the new host as pJLM400.

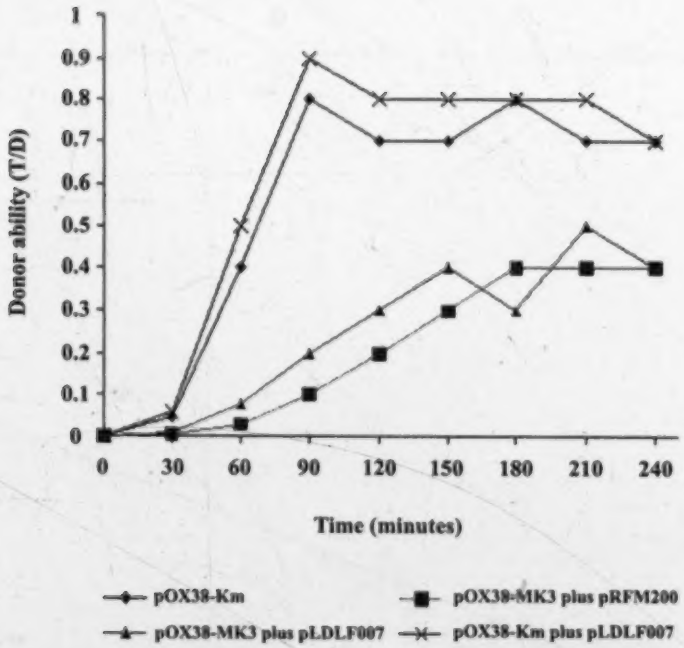
DNA sequencing was performed on all the plasmids from A1 to A5 to examine the sequence of *traM*, P_{lac} and the replicative origin (*oriV*) (Figure 3-4C). The replicative origins of the plasmids were identical to that in pJLM400, suggesting that changes in plasmid copy number did not result from mutations in *oriV*. All the plasmids contained a wild type *traM* gene and *lac* promoter region, whereas plasmids from A1, A2, A3, and A5 carried an extra sequence of 1199 base pairs between the ribosome-binding site and *traM*. A BLAST search of this sequence against the *E. coli* K12 genome sequence (Genbank Accession No. NC_000913) identified this as an IS*5* insertion sequence from the *E. coli* K12 chromosome (Kroger and Hobòm, 1982). IS*5* had inserted into a CTAA sequence between the ribosome-binding site and *traM*, resulting in a direct repeat of this motif on each side of the insertion. The IS*5* insertion also appeared to affect plasmid copy number, since plasmids from A1, 2, 3, and 5 did not reach the copy number of the parental plasmid (pJLM400) in the new host.

The results indicated that the adapted strain A4 reduced TraM expression through unknown host mutations to reduce the copy number of *traM*, whereas A1, A2, A3, and A5 abolished TraM expression through an IS*5* insertion in the plasmid.

3.2.4 Role of P_{traM} in F conjugation when F^+ cells recover from stationary phase

TraM is potentially toxic but the exceptionally strong P_{traM} is maintained in the F plasmid through evolution, implying that a strong P_{traM} is beneficial to the F plasmid.

Figure 3-5. Donor ability of cells containing pOX38-Km or pOX38-MK3 in the presence of TraM supplied by pRFM200 or pLDLF007 during recovery from stationary phase. Donor cultures were grown for 20 hours and diluted into fresh medium at a 1:100 ratio. During growth with vigorous aeration at 37°C, aliquots of donor cells were taken at 30-minute intervals over the time course and added to fresh ED24 recipient cells for 30 minutes and assayed for donor ability. T/D represents transconjugants per donor.


Donor ability (T/D)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
30
60
90
120
150
180
210
240
Time (minutes)
pOX38-Km
pOX38-MK3 plus pRFM200
pOX38-MK3 plus pLDLF007
pOX38-Km plus pLDLF007

Although P_{traM} is normally repressed by TraM, stationary-phase F^+ cells do not express detectable levels of TraM (Frost and Manchak, 1998). Therefore, when F^+ cells are recovering from stationary phase, P_{traM} is not repressed until intracellular concentrations of TraM reach a threshold. Presumably, P_{traM} is not repressed during the early stage of establishment in new transconjugants either. To determine the importance of the strong P_{traM}, I monitored the recovery of the donor ability of cells containing pOX38-Km (a wild type F derivative) or cells containing pOX38-MK3 complemented by pLDLF007 or pRFM200 (Figure 3-5).

After incubation for 20 hours, donor ability of each culture was undetectable as expected. Upon a 1:100 dilution into fresh medium, cells containing pOX38-Km regained maximum donor ability after 90 minutes, whereas cells containing pOX38-MK3 plus pLDLF007 or pRFM200, reached maximum donor ability after 150 or 180 minutes, respectively. The growth rates of these cultures were similar (data not shown). pLDLF007, which expresses *traM* from P_{traM} as pOX38-Km, could not complement pOX38-MK3 to the same levels of transfer frequency as pOX38-Km. pRFM200, which expresses *traM* from an attenuated version of P_{traJ} (Chapter 4), did not complement pOX38-MK3 as well as pLDLF007. These results suggested that transcription of *traM* from P_{traM} *in cis* to *traJ* and the major *tra* operon facilitated F plasmid transfer as host cells recovered from stationary phase.

3.2.5 Role of P_{traM} in epidemic spread of FinOP-inhibited pOX38-Km

Strong promoters have also been found in transfer-repressed F-like plasmids R1 and R100 (Abo and Ohtsubo, 1993; Schwab *et al.*, 1993). During the early stage of new

transconjugants, transcription from the unregulated P_{traM} might increase *traJ* transcription significantly via readthrough into *traJ*. Dempsey (1989) has demonstrated that expressing extra copies of the *traJ* leader sequence *in trans* can counteract FinOP inhibition to de-repress conjugative transfer of R100. Here, pSnO104, which expresses FinO from an IncFII plasmid R6, reduced the transfer frequency of the co-resident pOX38-Km by nearly 200-fold and reduced TraJ to undetectable levels through restoration of FinOP inhibition (Table 3-1; Figure 3-7A lane 10). When extra copies of the *traJ* leader sequence from the F plasmid were expressed by pJLJ001 *in trans*, the transfer frequency of pOX38-Km increased two logs in the presence of FinOP inhibition (Table 3-1); and TraJ could be detected using anti-TraJ antiserum (Figure 3-7A lane 11). This indicated that extra transcripts of F *traJ* 5' UTR (untranslated region) counteract FinOP inhibition, allowing de-repressed TraJ expression and F conjugation. pJLJF001, which is capable of expressing both the *traJ* leader sequence and *finP*, de-repressed the pOX38-Km in the presence of FinO at slightly lower levels than pJLJ001.

In new transconjugants, increased levels of *traJ* transcripts due to transcriptional readthrough from the unregulated P_{traM} could also counteract FinOP inhibition thereby allowing epidemic spread of naturally transfer-repressed F-like plasmids. In order to examine this possibility, the importance of P_{traM} in potential epidemic spread of FinOP-inhibited pOX38-Km was assayed (Materials and Methods; Figure 3-6). F^+ cells (pOX38-Km/pSnO104; $FinO^+$) were mixed with F^- cells (pT74 / pSnO104; $FinO^+$) at a 15:100 ratio and incubated at 37°C for 18 hours without shaking (Figure 3-6A). The number of F^+ cells increased more than 11-fold to 5.2×10^8, whereas the number of F^- cells was reduced 40% to 1.8×10^8 despite that the total cell number was doubled after 18 hours,

Table 3-1. Effect of supplying the untranslated region of *traJ* (*traJ* 5' UTR) on pOX38-Km in the presence of FinO supplied by pSnO104

Co-resident plasmid[a]	None	pACYC184	pSnO104	pSnO104 + pJLJ001	pSnO104 + pJLJF001	pSnO104 + pT7-5
Description of donors	F^+	F^+	F^+ $FinO^+$	F^+ $FinO^+$, extra *traJ* 5' UTR	F^+ $FinO^+$, extra *traJ* 5' UTR and *finP*	F^+ $FinO^+$
Transfer efficiency[b]	5×10^{-1}	5×10^{-1}	3×10^{-3}	4×10^{-1}	2×10^{-1}	3×10^{-3}

[a] pJLJ001 expresses the 5' UTR of *traJ* from P_{traJ}; pJLJF001 expresses the 5' UTR of *traJ* and FinP from P_{traJ} and P_{finP}, respectively. PACYC184 is the vector control for pSnO104 whereas pT7-5 is the vector control for pJLJ001 and pJLJF001.

[b] Transfer efficiency is calculated as the number of transconjugants per donor cell.

indicating that a majority of F^+ cells are new transconjugants. The donor ability of the F^+ cells was 0.17 transconjugants per donor. As a control, F^+ cells that were incubated alone for 18 hours had a donor ability at 0.003 transconjugants per donor (Figure 3-6C). These results suggested that FinOP-inhibited pOX38-Km transfers at nearly 60-fold higher levels from new transconjugants to F^- recipients than from the original donors, which is characteristic of epidemic spread.

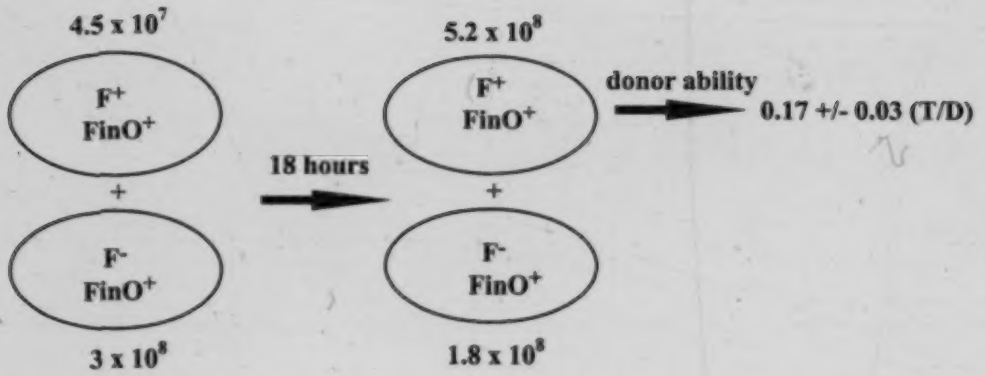
To repress P_{traM} of pOX38-Km in new transconjugants, TraM was expressed by pLDLF007 in F^- cells. F^+ cells (pOX38-Km/pSnO104; $FinO^+$) were mixed with F^- cells (pLDLF007 / pSnO104; $TraM^+$ $FinO^+$) at a 15:100 ratio and incubated at 37°C for 18 hours without shaking (Figure 3-6B). The total cell number doubled and the number of F^+ cells (4.7×10^8) increased more than 10-fold, whereas the number of F^- cells decreased 20%. The donor ability of the F^+ cells was 0.05 transconjugants per donor (Figure 3-6B), nearly 17-fold higher than that of the donor control but approximately 3.5-fold lower than that of the cells without pLDLF007 (Figure 3-6A and C). Therefore, supplying TraM in recipient cells appeared to allow repression of P_{traM} in new transconjugants thereby decreasing levels of epidemic spread, suggesting that P_{traM} facilitated epidemic spread of the transfer-repressed F-like plasmids.

3.2.6 Relation of TraM, TraJ, TraY, and TraA expression

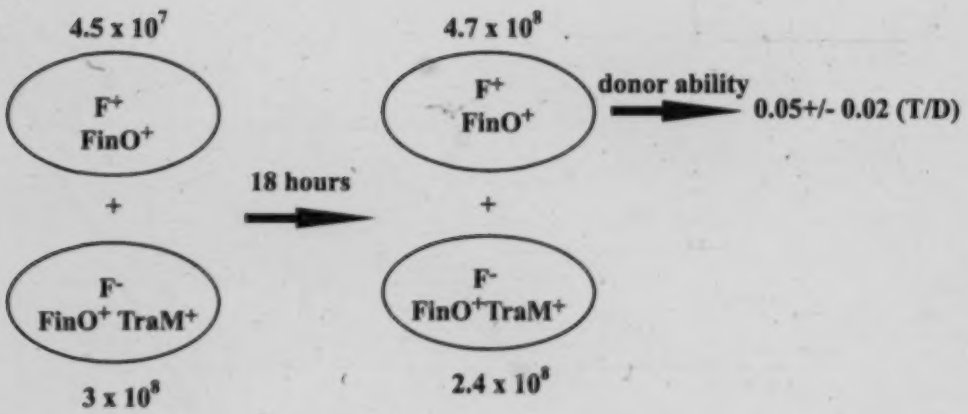
F plasmid TraJ is an essential positive regulator for the major *tra* operon containing *traY* (Cuozzo and Silverman, 1986), whereas TraY is required for *traM* transcription that might increase *traJ* expression through transcriptional readthrough (Penfold *et al.*, 1996;

Figure 3-6. Epidemic spread of transfer-repressed pOX38-Km. (A) Epidemic spread of transfer-repressed pOX38-Km. (B) Epidemic spread of transfer-repressed pOX38-Km with TraM expressed in the initial recipients. (C) Donor ability of transfer-repressed pOX38-Km incubated alone for 18 hours. Ovals represent XK1200 cells with the numbers of cells indicated above or below. F^+ represents pOX38-Km. $FinO^+$ represents pSnO104. $TraM^+$ represents pLDLF007. The donor ability of the 18-hour XK1200 cell mixtures was assayed by mixing 0.05 ml of the 18-hour cell mixture with 0.2 ml of fresh, late-exponential phase ED24 cells in 1 ml of LB broth and following the standard procedure as described in Material and Methods. Donor ability was calculated as the number of F^+ ED24 transconjugants (Km^RSpc^R) divided by the number of F^+FinO^+ XK1200 cells ($Km^RCm^RNal^R$). T/D represents transconjugants per donor.

(A)


4.5 x 10^7
F+ FinO+
+
F- FinO+
3 x 10^8
18 hours
5.2 x 10^8
F+ FinO+
+
F- FinO+
1.8 x 10^8
donor ability
0.17 +/- 0.03 (T/D)

(B)


4.5 x 10^7
F+ FinO+
+
F- FinO+ TraM+
3 x 10^8
18 hours
4.7 x 10^8
F+ FinO+
+
F- FinO+TraM+
2.4 x 10^8
donor ability
0.05+/- 0.02 (T/D)

(C)

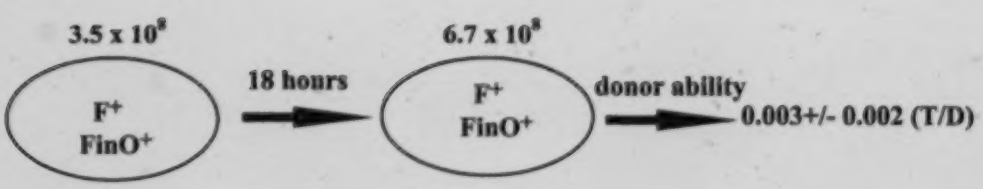

3.5 x 10^8
F+ FinO+
18 hours
6.7 x 10^8
F+ FinO+
donor ability
0.003+/- 0.002 (T/D)

Figure 3-1B). To further understand this complicated circuit of regulation, I assayed levels of TraM, TraJ, and the first two proteins (TraY and TraA) encoded by the major *tra* operon under different conditions.

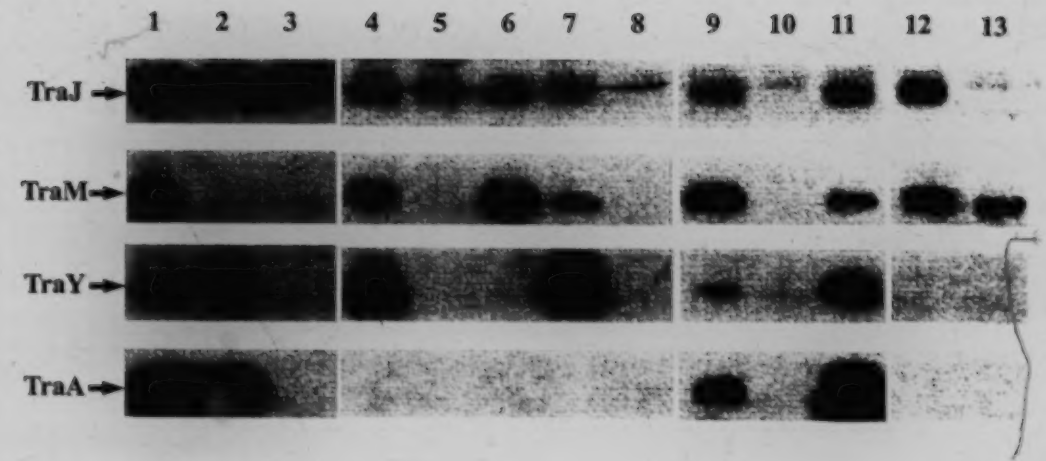
To further study the importance of TraY for *tra* gene expression, a *traY*-disrupted F-derivative, pOX38-*traY244* was used. pOX38-*traY244* expressed undetectable levels of TraM, TraA (pilin) and lowered levels of TraJ (Figure 3-7A lane 1, 3, and 5). Expression of TraM by pLDLF007 was unaffected in the presence of pOX38-*traY244* (Figure 3-7B lane 6), indicating that the F plasmid did not encode any other *in trans* factors that affected expression of *traM* in the absence of TraY. When TraY was supplied *in trans*, pOX38-*traY244* expressed levels of TraM and TraJ comparable to pOX38-Km (Figure 3-7A lane 4), indicating that TraY is required for expression of both *traM* and *traJ*. TraA remained undetectable, suggesting that the disruption of *traY* in pOX38-*traY244* had polar effects on downstream genes.

Unlike TraJ that directly activates P_{traY} (Figure 3-1B; Mullineaux and Willetts, 1985), TraY did not appear to directly increase the strength of P_{traM} or transcriptional readthrough into *traJ* (pJLac111, pJLac115, and pJLac119; Figure 3-1B). When cloned in pJLac101, P_{traM} and P_{traJ} were both active in the absence of TraY (Figure 3-1B). Similarly, pJLac113, which contains an F fragment from *oriT* to the beginning of *traY* (Figure 3-1B), expressed normal levels of TraM and TraJ (Figure 3-7A lane 12). Therefore, dependence of *traM* and *traJ* on TraY appeared to exist only in the context of the F plasmid.

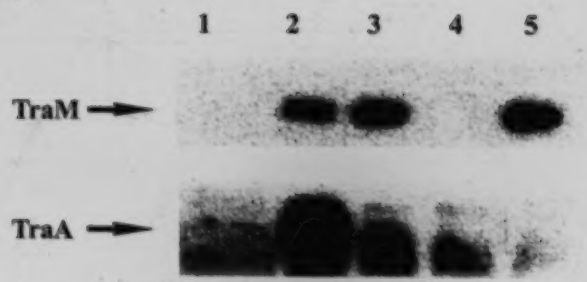
FinO has been found to repress *traM* in the F plasmid (Penfold *et al.*, 1996). Since

Figure 3-7. Levels of different *tra* gene products. Each lane was loaded with 0.1 OD_{600} of cells containing different plasmids as indicated. Proteins were detected by immunoblot analysis using corresponding antisera as described in Materials and Methods. Arabinose was added in the culture of cells containing pBADTraY or pBAD24 at a final concentration of 0.1% (W/V). (A) Relation of TraM, TraY, TraJ and TraA expression. Lane 1: pOX38-Km; lane 2: pOX38-MK3; lane 3: pOX38-*traY244*; lane 4: pOX38-*traY244* and pBADTraY; lane 5: pOX38- *traY244* and pT74; lane 6: pOX38- *traY244* and pLDLF007; lane 7, pBADTraY, pOX38-Km and pSnO104; lane 8: pOX38-Km, pSnO104, and pBAD24; lane 9: pOX38-Km, pSnO104, and pLDLF007; lane 10: pOX38-Km, pSnO104, and pT7-4; lane 11: pOX38-Km, pSnO104, and pJLJ001; lane 12: pJLac113 and pACYC177; lane 13: pJLac113 and pED104. (B) Effects of TraM on TraA when *traJ* was present or absent. Lane 1: JCFL90; lane 2: JCFL90 and pJLac114; lane 3: JCFL90 and pLDLF007; lane 4: JCFL90 and pT7-4; lane 5: pLDLF007.

(A)



(B)



FinOP represses TraJ expression thereby repressing TraY expression, the effect of FinO on TraM expression could be due to lack of TraY. In fact, when FinO was supplied *in trans*, pJLac113 expressed TraM but not TraJ (Figure 3-7A lane 13), whereas pOX38-Km did not express detectable levels of TraM, TraJ, TraY, or TraA (Figure 3-7A lane 8 and 10). This indicated that FinO directly repressed TraJ expression but repressed TraM expression only in the context of the F plasmid, which correlates with the way TraY is required for TraM expression. When TraY was supplied *in trans*, expression of TraM and TraJ but not TraA was partially restored in pOX38-Km/pSnO104 (Figure 3-7A lane 7), further suggesting that FinO represses TraM expression indirectly through repressing TraY expression in the F plasmid.

Unexpectedly, TraM appeared to be able to partially de-repress FinOP-inhibited pOX38-Km. When TraM was supplied *in trans*, pOX38-*traY244* expressed detectable levels of TraJ, and pOX38-Km/pSnO104 partially resumed expression of TraJ, TraY and TraA (Figure 3-7A lane 6 and 9). However, TraM did not appear to be essential for expression of TraJ, TraY or TraA, since pOX38-MK3 expressed levels of TraJ, TraY and TraA comparable to pOX38-Km (Figure 3-7A lane 1 and 2). Since TraJ is a positive regulator for the major *tra* operon, the effect of TraM on TraY and TraA could be due to the increase of TraJ expression. In fact, when TraM was supplied by the co-resident plasmid pLDLF007, JCFL90, a *traJ*-deficient F'*lac* plasmid that does not normally express TraM or TraA (Figure 3-7B lane 1), did not resume TraA expression (Figure 3-7B lane 3 and 4). Therefore, TraM did not appear to increase TraA expression without TraJ. As a control, when TraJ was supplied *in trans* by pJLac114 (a *traM*-disrupted

pJLac113 derivative), JCFL90 resumed expression of TraM and TraA (Figure 3-7B lane 2), indicating that expression of *traM* and *traA* in the F plasmid requires TraJ.

3.3 Discussion

P_{traM} is exceptionally strong but stringently autoregulated such that the potential cytotoxicity of TraM is controlled. This raises the question as to the benefit of such a strong promoter in the F plasmid. P_{traM} *in cis* to the *tra* operons appeared to be required for efficient F conjugation when host cells recover from stationary phase or during establishment of the F plasmid in new transconjugants (Figure 3-5; Figure 3-6). The F plasmid benefits by efficiently spreading throughout an F⁻ cell population thereby ensuring plasmid survival (Bingle and Thomas, 2001). One explanation for the F plasmid maintaining such a strong P_{traM} is that unregulated transcription of *traM* efficiently increases the level of TraM, an essential protein for F conjugation. However, pLDLF007, which expresses *traM* from P_{traM}, did not complement pOX38-MK3 to the same level as pOX38-Km (Figure 3-5). This result agrees with previous observations in R1 and R100 plasmids (Dempsey, 1994; Polzleitner *et al.*, 1997), suggesting that the levels of TraM are not the limiting factor for maximum transfer ability. On the other hand, TraJ rather than TraM appears to be the limiting factor in new transconjugants.

Stockwell and Dempsey (1997) have shown that most transcripts originating from *traM* in R100 pass the *traM-traJ* intergenic region into *traJ*, with the *traM* internal promoters (P_{int}) playing a large role in this process. Transcripts corresponding to the *traM-traJ* dicistronic mRNA has also been detected in the F plasmid using probes for *traJ* or *traM* (Lee *et al.*, 1992; R. Will and L. S. Frost, unpublished data). Approximately two thirds of transcription from *traM* passed T_{traM} into *traJ* as determined by transcriptional fusion analysis (Figure 3-1D). The ratio of P_{int} and P_{traM} in strength was approximately 1:17, whereas this ratio is nearly 1:5 for R100 (Stockwell and Dempsey,

1997), suggesting that P_{int} in the F plasmid is not as important as in R100. Transcriptional readthrough from the repressed P_{traM} plus P_{int} (pJLac109) was approximately 20% of P_{traJ} (pJLac106) in strength and thus did not increase the total strength of P_{traJ} significantly (pJLac110 compared to pJLac106 in Figure 3-1 B). However, when in new transconjugants or when F^+ cells are recovering from stationary phase, P_{traM} is not fully repressed until enough TraM is expressed to establish autoregulation. Since P_{traM} was more than 3-fold stronger than P_{traJ} (Figure 3-1B), transcription from the unregulated P_{traM} could significantly increase transcription of *traJ*. Enhanced expression of TraJ, the positive regulator for the major *tra* operon, could then facilitate F conjugation. Taken together, our data and data presented by other groups (Dempsey, 1994; Polzleitner *et al.*, 1997) suggest that transcriptional readthrough into *traJ* allows *traM* to positively regulate *traJ* and the major *tra* operon in F and F-like plasmids. This mechanism ensures maximal transfer of the F plasmid and facilitates epidemic spread of the naturally transfer-repressed F-like plasmids.

TraM not only binds to its cognate sites near *oriT* but also binds nonspecifically to other sites on DNA when present at high protein concentrations (Fekete and Frost, 2002). High levels of TraM might non-specifically bind to the host chromosome to interfere with DNA replication and partitioning, resulting in cytotoxicity. The low-copy pJLM501, which mimics the F plasmid expressing *traM* from unregulated P_{traM}, resulted in decreased growth rate of host cells and reduced levels of F conjugation, which are disadvantages for competing with other cells in natural environment. When TraM was overexpressed by the high-copy pJLM400, the survived *recA* host cells (DH5α) either mutated to reduce the plamid copy number and TraM expression or had an IS*5*

transposition to inactivate *traM*. Presumably, more mutation or recombination events could happen to inactivate *traM* thereby avoiding TraM overexpression in wild type strains. However, for the very low-copy F plasmid, a further lowering of the copy number is impossible, whereas inactivation of *traM* would abolish F conjugation.

TraY is required for *traM* transcription in F and F-like plasmids (Penfold *et al.*, 1996; Stockwell and Dempsey, 1997; Polzleitner *et al.*, 1997). However, the activity of P_{traM} or expression of TraM appeared to require TraY only in the context of the F plasmid (Figure 3-1; Figure 3-7A). Since the F plasmid does not encode any factors that affect TraM expression *in trans* (Figure 3-7B), TraY appears to be a de-repressor that releases P_{traM} from an unknown repression in an *in cis* manner. In a similar way, TraM expression was TraJ-dependent and subject to FinOP repression. These observations could be explained by a hypothesis that in the absence of TraY, the F plasmid forms a "closed" structure involving extensive regions of the plasmid (Chapter 7). Transcription of *traM* and *traJ* is repressed in this closed structure. TraY is essential for disrupting this structure presumably by binding to its cognates sites upstream of P_{traM}. Therefore, disruption of *traJ* or supplying FinO to repress TraJ expression prevents TraY expression, allowing formation of the closed structure to repress *traM* expression. Although TraM is not essential for disrupting the closed structure, binding of TraM to *oriT* might also disrupt the structure, partially de-repressing *traJ* in pOX38-*traY244* or in FinOP-repressed pOX38-Km (Figure 3-7A).

The F plasmid also appears to have other mechanisms to regulate expression of *tra* genes. Deletion of P_{finP} more than doubled the strength of P_{traJ}, agreeing with the previous observation that deletion of *finP* increases the total strength of P_{traM} and P_{traJ} in

the R1 plasmid (Schwab *et al.*, 1993). This effect was not reversed by supplying FinP *in trans* (Schwab *et al.*, 1993), suggesting that *finP* transcription has an *in cis* negative effect on *traJ* transcription. This explains a previous observation that a mutation disrupting P_{finP} in the F plasmid increases *traJ* transcription significantly (Lee *et al.*, 1992). Interestingly, supplying TraY *in trans* partially de-repressed FinOP-inhibited pOX38-Km, allowing expression of TraJ but not TraA (Figure 3-7A). This suggests that extra TraY repressed the major *tra* operon, agreeing with the observation that TraY of R100 is a repressor for the major *tra* operon (Taki *et al.*, 1998) but arguing against the suggestion that TraY is an activator for P_{traY} (Silverman and Sholl, 1996).

Repression of P_{traM} by pLDLF007 in new transconjugants resulted in lowered levels of epidemic spread of transfer-repressed pOX38-Km, suggesting that unregulated *traM* transcription from P_{traM} facilitates epidemic spread of transfer-repressed F-like plasmids (Figure 3-6). As discussed above, transcriptional readthrough from the unregulated P_{traM} could significantly increase *traJ* transcription thereby allowing efficient expression of *tra* gene products before establishment of FinOP inhibition in new transconjugants. Furthermore, increased *traJ* transcripts might also counteract FinOP inhibition (Table 3-1; Dempsey, 1989). This explains why strong P_{traM} is conserved in transfer-repressed F-like plasmids (Abo and Ohtsubo, 1993; Schwab *et al.*, 1993). Since P_{traM} overlaps TraM binding sites, which are important for F conjugation (Fu *et al.*, 1991), it is difficult to alter P_{traM} without affecting F conjugation. pLDLF007 was used to express TraM in recipients thereby repressing P_{traM} in new transconjugants (Figure 3-6). However, supplying TraM *in trans* might have counteracted repression of P_{traM} since TraM could partially de-repress FinOP-inhibited pOX38-Km (Figure 3-7) and increase its transfer

efficiency by approximately 3-fold (data not shown). This could be one of the reasons that a substantial level of epidemic spread remained when P_{traM} was repressed by pLDLF007 in new transconjugants (Figure 3-6B).

In conclusion, strong F *traM* promoters facilitate F conjugation thereby advantageous for plasmid survival in bacteria population. On the other side, autoregulation of P_{traM} appears to be an effective way to prevent potential TraM toxicity and genetic mutation of *traM* without sacrificing the promoter strength of *traM*. There appears to be an *in cis* mechanism in F and F-like plasmids that might involve formation of a closed structure at P_{traM} in the absence of TraY (chapter 7). TraY is an essential de-repressor for releasing this inhibition thereby coordinating expression of the major *tra* operon with that of *traM* and *traJ*. This *in cis* inhibition mechanism acts together with FinOP inhibition to repress all the *tra* genes in repressed F-like plasmids. Low levels of TraY might de-repress *traM* in the closed conformation; strong transcription of *traM* would then facilitate *traJ* transcription via transcriptional readthrough, thus further increasing transcription of the major *tra* operon including *traY*. Increased expression of *traM* and *traY* might further disrupt the closed structure, allowing maximal expression of *traJ* and the major *tra* operon. Finally, autoregulation of *traM* and the major *tra* operon prevents unnecessary overexpression.

Chapter 4

A Rapid Screen for Autoregulation-Defective TraM Mutants

• A version of this chapter has been published: Lu, J., R. A. Fekete, and L. S. Frost. 2003. A rapid screen for functional mutants of TraM, an autoregulatory protein required for F conjugation. Mol. Gen. Genomics **269**:227-233.

4.1 Introduction

TraM is required for conjugative transfer of the F and F-like plasmids, a large group of plasmids that carry genes for antibiotic resistance and toxin production. TraM exists mainly as tetramers and binds specifically to its cognate sites cooperatively (Abo *et al.*, 1991; Schwab *et al.*, 1991; Di Laurenzio *et al.*, 1992; Verdino *et al.*, 1999; Fekete and Frost, 2002). TraM has been found to interact with an inner membrane protein, TraD (Disque-Kochem and Dreiseikelmann, 1997), supporting the hypothesis that TraM performs a signaling function during the conjugation process (Willetts and Wilkins, 1984).

In the F plasmid, P_{traM} includes two separate promoters, which overlap two of the TraM binding sites, *sbmA* and *sbmB* (Di Laurenzio *et al.*, 1992; Penfold *et al.*, 1996). P_{traM} is exceptionally strong but is stringently repressed by TraM (Chapter 3). The strong, autoregulated nature of P_{traM} poses a problem for *in situ* mutational analysis of *traM*. Mutations that affect autoregulation to differing degrees could result in differing levels of *traM* overexpression from P_{traM}, making it difficult to compare their biological effects *in vivo*. Furthermore, as high levels of TraM have been found to be toxic to host cells (Chapter 3), certain autoregulation-defective mutants might cause affect cell death.

In this work, *traM* mutants were generated by random PCR mutagenesis and the mutated *traM* was expressed at a low, functional level from a constitutive promoter. Based on the autoregulatory function of TraM, a P_{traM}-*lacZ* fusion with a -1 frameshift mutation was used to isolate autoregulation-defective *traM* mutants. Selected TraM mutants were further characterized for their intracellular levels and activity for autoregulation and F conjugation.

4.2 Results

4.2.1 Constitutive expression of *traM* and its mutants

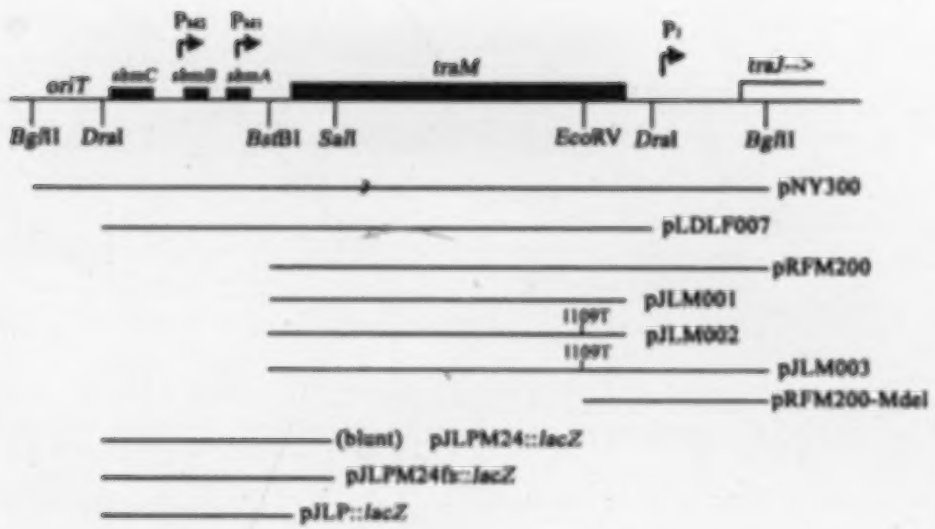
Constitutive expression of *traM* and its mutants could be achieved by supplying the strong, constitutive P_{traJ} after *traM* and letting it transcribe around the entire plasmid in such a way that the distance from the promoter attenuates its strength and gives low levels of TraM. Therefore, pRFM200 was constructed by cloning *traM* and P_{traJ} but without P_{traM} in pT7-5 (Figure 4-1A).

The level of TraM expressed by pRFM200 was assayed by immunoblot and was compared to that of the single-copy F derivative, pOX38-Km. In the absence of T7 polymerase, pRFM200 constitutively expressed a level of TraM comparable to pOX38-Km (Figure 4-1B). pJLM001, which is identical to pRFM200 except that it lacks P_{traJ} (Figure 4-1A), did not express detectable TraM, indicating that P_{traJ} is the major promoter for the constitutive expression of *traM*. To ensure that *traM* mutants could be expressed at levels comparable to wild-type *traM* (wt-*traM*) in pRFM200, pJLM003 was constructed. pJLM003 is equivalent to pRFM200 but contains the *traM* point mutation I109T instead of wt-*traM*. pJLM003 expressed a level of TraMI109T comparable to that for pRFM200. Deletion of P_{traJ} (pJLM002) also prevented expression of TraMI109T. As a control, pRFM200-Mdel, a pRFM200 derivative with a deletion in *traM* but with P_{traJ} intact, did not express detectable TraM.

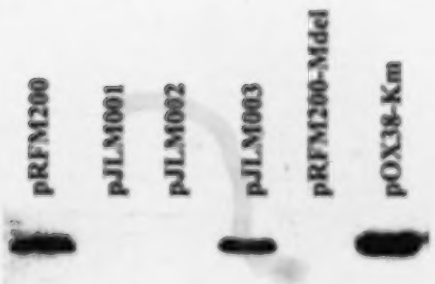
pRFM200 complemented the *traM*-deficient plasmid pOX38-MK3 and restored its transfer ability to a level comparable to that of pOX38-Km, while pJLM003 (*traM*I109T) was highly deficient for complementation in conjugation (Table 4-2). Thus, constitutive

Figure 4-1. (A) F fragments cloned in different constructs. P_{M1} and P_{M2} are the *traM* promoters (P_{traM}); *sbmA*, *-B*, *-C* are the three TraM binding sites within *oriT*; P_J is P_{traJ}; each line represents a corresponding F fragment cloned in a construct indicated at the right end of the line. Restriction sites are below the line; angled arrows indicate direction and location of promoters. The *traM* and *traJ* genes are above the line; the TraM coding region is indicated with a block box; the incomplete coding region of *traJ* is an open white box. I109T refers to the position of the missense mutation. The abbreviations used are list in the "Abbreviations" section. (B) Levels of TraM and its mutants expressed by various plasmids. Each lane was loaded with 0.1 OD_{600} of cells containing different plasmids as indicated. TraM was detected by immunoblot analysis using anti-TraM antiserum as described in Materials and Methods.

(A)


oriT
shmC
shmB
shmA
traM
traJ-->
BglII
DraI
BstBI
SalI
EcoRV
DraI
BglII
pNY300
pLDLF007
pRFM200
pJLM001
1109T
pJLM002
1109T
pJLM003
pRFM200-Mdel
(blunt) pJLPM24::lacZ
pJLPM24fr::lacZ
pJLP::lacZ

(B)


pRFM200
pJLM001
pJLM002
pJLM003
pRFM200-Mdel
pOX38-Km

expression from P_{traJ} of *traM* or various *traM* mutants gave the same intracellular level of proteins and allowed comparison of their functional differences.

4.2.2 Development of a screening system for *traM* mutants

In order to find a suitable reporter plasmid to select autoregulation-defective *traM* mutants, pJLPM24::*lacZ*, which contains P_{traM} upstream of the *lacZ* reporter gene was constructed. The ribosome binding site and first 24 codons of *traM* were fused in-frame with *lacZ* supplied by pPR9tt. pJLP::*lacZ* was identical to pJLPM24::*lacZ* but lacked the *traM* sequence after the start codon. It expressed a level of β-galactosidase activity similar to that for pJLPM24::*lacZ* (Table 4-1). This indicated that the first 24 residues of TraM could not repress P_{traM} and agreed with the previous finding that first 22-amino acid peptide of R1 TraM did not bind to its binding sites (Schwab *et al.*, 1993).

Host cells containing pJLPM24::*lacZ* expressed very high levels of β-galactosidase (Table 4-1), and formed dark blue colonies on BCIG-containing solid medium. Cells containing pJLPM24::*lacZ* and pOX38-Km, which supplied TraM, formed dark blue colonies similar to those formed by cells containing pJLPM24::*lacZ* and pOX38-MK3 which lacks *traM*. Because of the strength of P_{traM} in pJLPM24::*lacZ*, colonies were dark blue whether or not the promoter was repressed by TraM, indicating that pJLPM24::*lacZ* was not able to differentiate *traM*-deficient cells from cells containing wt-*traM* by the color intensity of colonies.

Thus, a frameshift mutation was used to downregulate *lacZ* expression from the strong P_{traM}. Frameshifting happens at low levels during transcription and translation (Kurland, 1992) such that mutants that have a -1 frameshift mutation in the coding

Table 4-1. β-galactosidase activity expressed by DH5α cells containing different reporter plasmids

Reporter plasmid	Coresident plasmid	β-galactosidase Activity (MU)
pJLPM24::*lacZ*	none	18800 ±1900
pJLP::*lacZ*	none	19600 ±2100
pJLPM24fs::*lacZ*	none	34.8 ±1.9
pACPM24fs::*lacZ*	none	40.2 ±2.8
pACPM24fs::*lacZ*	pRFM200-Mdel	41.8 ±1.7
pACPM24fs::*lacZ*	pRFM200	8.3 ±0.7
pACPM24fs::*lacZ*	pJLM003	33.4 ±1.2

sequence are correctly transcribed or translated at a low level. pJLPM24fs::*lacZ* is identical to pJLPM24::*lacZ* except that the first 24 codons of *traM* were fused to *lacZ* in a -1 frameshift. This frameshift mutation decreased β-galactosidase expression by about 500-fold (Table 4-1). Cells containing pJLPM24fs::*lacZ* and pOX38-MK3 formed dark blue colonies on BCIG-containing solid medium, while significantly lighter blue colonies were obtained with cells containing pACPM24fs::*lacZ* and pOX38-Km. These results suggested that *traM*-deficient mutants could be selected by the color intensity of colonies carrying pJLPM24fs::*lacZ*. Also, autoregulation-defective *traM* mutants could be differentiated from the wt-*traM*.

Since both pJLPM24fs::*lacZ* and pRFM200 contain the ampicillin resistance gene, a fragment containing P_{traM} and the frameshift fusion from pJLPM24fs::*lacZ* was cloned into pACYC184 (containing chloramphenicol resistance), resulting in pACPM24fs::*lacZ* , to allow antibiotic selection for both pRFM200 and the reporter plasmid. Cells containing the resulting plasmid (pACPM24fs::*lacZ*) and pRFM200 expressed β-galactosidase activity at 8.3 MU (Table 4-1), and formed light blue colonies (Figure 4-2A), whereas cells containing pACPM24fs::*lacZ* and pRFM200-Mdel expressed increased levels of β-galactosidase activity (41.8 MU) comparable to cells containing pACPM24fs::*lacZ* alone. When pJLM003 (I109T) was transformed into cells containing pACPM24fs::*lacZ*, β-galactosidase activity was not repressed significantly (33.4 MU), and the transformants formed dark blue colonies similar to those formed by cells containing pACPM24fs::*lacZ* and pRFM200-Mdel. Thus, simply by the color intensity of the colonies, autoregulation-defective *traM* mutants could be selected in the presence of the reporter plasmid, pACPM24fs::*lacZ*.

4.2.3 Selection of *traM* mutants generated by random PCR mutagenesis

To generate mutations in *traM*, error-prone PCR was used to amplify *traM* from pRFM200 under conditions described in Materials and Methods. The products were cloned into pT7-5 as for pRFM200, and were then transformed into cells containing pACPM24fs::*lacZ*. A total of 234 blue colonies were selected from approximately 25,000 transformants. Sequence analysis revealed 135 pRFM200 derivatives with point mutations in *traM*, whereas the remaining colonies contained frameshift mutations, multiple point mutations, or mutations within the ribosome binding site or start codon of *traM*, which were discarded. The135 point mutations, including 72 missense and 4 nonsense mutations with 59 redundant, were located in 56 different residues of TraM (Table 4-2).

4.2.4 Preliminary characterization of *traM* mutants

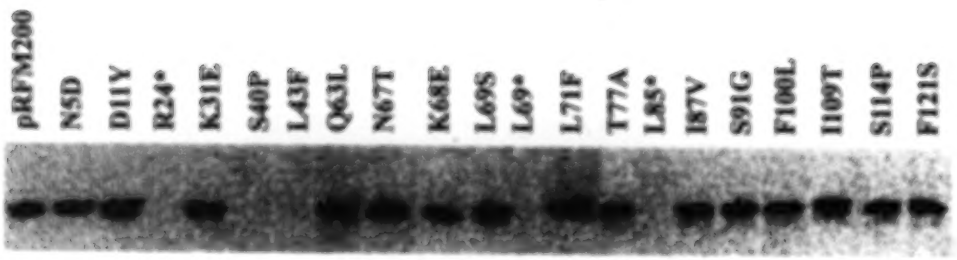
To assay the levels of autoregulation, the β-galactosidase activity of the colonies containing pRFM200 derivatives and pACPM24fs::*lacZ* were determined. The β-galactosidase activity of the colonies containing single point mutations in *traM* ranged from 18 MU to 45 MU (Table 4-2), whereas cells containing pRFM200 (wild type) or pRFM200-Mdel (a *traM*-deleted pRFM200 derivative) was 8 MU or 42MU, respectively. This indicated that different point mutations resulted in different levels of autoregulation. Except for nonsense mutations, the cells with β-galactosidase activity above 40 MU contained N-terminal point mutations in *traM*.

Figure 4-2. Expression of TraM and its mutants. (A) The color intensity of colonies containing pACPM24fs::*lacZ* and pRFM200 (TraM), pRFM200-Mdel (TraM⁻), or pJLM003 (I109T) on LB plates containing X-gal. (B) Levels of TraM and its mutants expressed by pRFM200 and its derivatives, respectively. Each lane was loaded with 0.1 OD_{600} of cells containing different plasmids as indicated. TraM was detected by immunoblot analysis using anti-TraM antiserum as described in Materials and Methods. pRFM200 derivatives are indicated above the figure by the names of the corresponding mutations.

(A)

pRFM200 pRFM200-Mdel pJLM003

(B)



To examine whether or not the lack of P_{traM} repression was due to decreased levels of mutated TraM, immunoblot analysis was used to assay the intracellular levels of the mutants. Most point mutations did not affect levels of TraM as determined by immunoblot analysis with anti-TraM antiserum (Figure 4-2B; Table 4-2). However, some mutants with N-terminal missense mutations were difficult to detect, and S79* was the only one of the four nonsense mutants that was detected using anti-TraM antiserum.

To assess the effects of these mutations on the function of TraM in F conjugation, pRFM200 or its derivatives were transformed into *E. coli* XK1200 cells containing a *traM*-deficient F derivative, pOX38-MK3. Donor ability assays showed that pRFM200 (*traM*) restored the transfer of pOX38-MK3 to a frequency at 4×10^{-1} transconjugants per donor, while none of the pRFM200 derivatives complemented pOX38-MK3 completely (Table 4-2).

4.3 Discussion

Random PCR-mutagenesis is a simple approach to create mutations in a target gene. This method takes advantage of the low fidelity of the *Taq* DNA polymerase (Tindall and Kunkel, 1988), and generates random small mutations, such as nucleotide substitutions. Providing there is an effective screen for selection of potential mutants, all amino acid residues within a protein that are essential for its function could be mapped. By using a low-fidelity DNA polymerase, excessive dNTPs, limited amount of the template, and an increased number of reaction cycles (Materials and Methods), replication errors were induced during PCR amplification of *traM*. Autoregulation-defective mutations were selected at a frequency of about 1%, and more than two thirds of the mutations were missense mutations that were useful for further analysis. However, this screen did not select silent mutations, mutations at permissive sites, or mutations affecting conjugation but not affecting autoregulation.

A -1 frameshift mutation in the reporter gene was employed as a simple solution for decreasing expression of the reporter gene and thus allowing visualization of differences in P_{traM} by color variation of the colonies between wild-type and mutant TraM expressed from a second compatible plasmid. During the normal translation process, frameshifting of the ribosome happens at a frequency of about 5×10^{-3} per codon (Kurland, 1992). Since the frameshift mutation was located at the interface between the first 24 codons of *traM* and the coding region of β-galactosidase in pACPM24fs::*lacZ*, any translation error causing the ribosome to move one nucleotide backwards could suppress the -1 frameshift mutation. This mechanism allowed the –1 frameshift fusion to express β-galactosidase at

a low level, which was suitable for visualizing differences between *traM* and *traM* mutants in repressing P_{traM}.

With 76 different point mutations (plus a 78% redundancy) covering 44% of TraM residues (Table 4-2), it is believed that a majority of the TraM residues important for autoregulation and F conjugation have been located. As mutations were selected from a large amount of randomly mutated *traM* fragments, the spread of these mutations in *traM* could reflect the domain conservation of TraM for its functions in autoregulation and F conjugation. As mutations affecting the functions for autoregulation and F conjugation are located throughout TraM, extensive regions in TraM appear to be involved in autoregulation and F conjugation, probably a reflection of the shared requirement of DNA binding and tetramerization for these two functions.

Regarding the autoregulation function of TraM, the N-terminal region of TraM appeared to be more important than the C-terminal region. When TraM mutants were constitutively expressed from pRFM200 derivatives, all the missense mutations causing complete loss of autoregulation (β-galactosidase activity > 40 MU) are located in the first 47 residues of TraM (Table 4-2). This agrees with the previous result that the first 24 residues of F-like R1 TraM define the specificity for autoregulation (Kuperlwieser *et al.*, 1998). However, residue substitutions throughout TraM affect autoregulation, suggesting that regions beyond the N-terminus are also important for autoregulation (Table 4-2). All these mutations also affected the function of TraM in conjugation, indicating an overlap of the structural determinants for these two functions of TraM. R24*, L69*, L85 *, and a few N-terminal mutants did not express detectable levels of protein, indicating that either

these mutations affected the stability of TraM, or the epitopes for the anti-TraM antiserum were lost due to truncation or residue substitution within the protein.

Thus, two simple solutions to the problems of controlling expression of a toxic protein and downregulating expression from an exceptionally strong promoter such as P_{traM}, were combined to allow the efficient isolation of important missense mutations in TraM. Further characterization of the mutants for DNA binding and oligomerization will be presented in Chapter 5.

Table 4-2. Properties of *traM* mutants[a] in pRFM200 derivatives

traM mutant	Codon change	Regulating P_{traM}, (MU)[b]	Detection of TraM [c]	Complementing pOX38-MK3[d]	*traM* mutant	Codon change	Regulating P_{traM}, (MU)[b]	Detection of TraM [c]	Complementing pOX38-MK3[d]
K3R	AAG to AGG	42.7±1.8	no	$< 1 \times 10^{-7}$	L69*	UUG to UAG	42.3±1.8	no	$< 1 \times 10^{-7}$
V4G	GUG to GGG	41.8±1.1	no	$< 1 \times 10^{-7}$	L69S	UUG to UCG	31.5±0.9	yes	5×10^{-2}
V4A	GUG to GCG	40.1±1.9	no	$< 1 \times 10^{-7}$	L69W	UUG to UGG	33.5±1.0	yes	1×10^{-2}
N5D	AAC to GAC	43.5±2.7	yes	$< 1 \times 10^{-7}$	L70P	CUU to CCU	37.1±1.5	yes	5×10^{-7}
Y7C	UAU to UGU	38.9±1.5	yes	$< 1 \times 10^{-7}$	L71F	CUU to UUU	25.5±1.0	yes	5×10^{-4}
I8N	AUC to AAC	43.1±0.9	no	$< 1 \times 10^{-7}$	T77A	ACA to GCA	32.5±1.0	yes	1×10^{-5}
S9C	AGC to UGC	30.5±1.1	yes (-)	5×10^{-2}	S79*	UCA to UAA	41.1±1.1	yes	$< 1 \times 10^{-7}$
N10T	AAU to ACU	39.2±1.2	yes (-)	$< 1 \times 10^{-7}$	S80P	UCA to CCA	36.6±1.2	yes	6×10^{-7}
N10D	AAU to GAU	38.7±0.9	yes (-)	5×10^{-7}	K83E	AAA to GAA	32.9±1.8	yes	5×10^{-7}
D11Y	GAU to UAU	25.3±0.6	yes	1×10^{-1}	K83N	AAA to AAC	30.1±2.3	yes	1×10^{-6}
Y13C	UAU to UGU	28.8±1.5	yes	1×10^{-2}	I84V	AUU to GUU	29.2±1.7	yes	1×10^{-5}
N17D	AAU to GAU	36.7±0.7	yes	5×10^{-5}	L85*	UUG to UAG	40.5±1.2	no	$< 1 \times 10^{-7}$
A18E	GCG to GAG	30.2±0.9	yes	5×10^{-2}	L85S	UUG to UCG	31.5±1.7	yes	$< 1 \times 10^{-7}$
I19F	AUU to UUU	38.5±1.1	yes (-)	3×10^{-5}	I87V	AUU to GUU	28.3±1.9	yes	8×10^{-5}

R23C	CGU to UGU	32.5±0.9	yes	1×10^{-4}	S89P	UCU to CCU	34.3±3.1	yes	8×10^{-7}
R24*	CGA to UGA	44.3±1.7	no	$< 1 \times 10^{-7}$	S89F	UCU to UUU	32.1±1.7	yes	$< 1 \times 10^{-7}$
R24Q	CGA to CAA	41.1±2.3	no	1×10^{-6}	S91G	AGU to GGU	33.8±1.1	yes	5×10^{-5}
E30G	GAA to GGA	21.9±0.8	yes	6×10^{-3}	S91C	AGU to UGU	28.8±2.2	yes	2×10^{-1}
K31E	AAA to GAA	44.4±2.0	yes	3×10^{-7}	P92R	CCU to CGU	23.3±1.9	yes	$< 1 \times 10^{-7}$
S34R	AGU to AGG	40.1±1.5	no	$< 1 \times 10^{-7}$	P92S	CCU to UCU	25.1±1.6	yes	1×10^{-3}
F35S	UUU to UCU	41.8±2.5	no	$< 1 \times 10^{-7}$	S95P	UCC to CCC	29.1±1.5	yes	2×10^{-6}
S36P	UCA to CCA	40.3±1.9	yes	$< 1 \times 10^{-7}$	F100L	UUU to CUU	27.0±1.4	yes	2×10^{-6}
A37V	GCA to GUA	37.5±2.2	yes	$< 1 \times 10^{-7}$	F100S	UUU to UCU	29.8±2.3	yes	2×10^{-7}
A37E	GCA to GAA	41.8±1.9	yes	$< 1 \times 10^{-7}$	Y102S	UAU to UCU	30.5±2.1	yes	$< 1 \times 10^{-7}$
A39T	GCU to ACU	30.8±2.1	yes	3×10^{-3}	N104D	AAU to GAU	30.2±1.7	yes	1×10^{-6}
S40P	UCA to CCA	41.4±0.5	no	$< 1 \times 10^{-7}$	M05T	AUG to ACG	29.9±1.2	yes	6×10^{-7}
S40L	UCA to UUA	36.5±1.9	yes (-)	$< 1 \times 10^{-7}$	M105V	ATG to GUG	26.1±0.5	yes	8×10^{-7}
M41T	AUG to ACG	28.3±1.6	yes	4×10^{-4}	I109T	AUC to ACC	32.8±0.6	yes	5×10^{-7}
L42P	CUU to CCU	43.3±2.9	no	$< 1 \times 10^{-7}$	S114P	UCA to CCA	29.5±0.6	yes	$< 1 \times 10^{-7}$
L43F	CUU to UUU	42.4±0.8	no	$< 1 \times 10^{-7}$	M117T	AUG to ACG	25.1±1.9	yes	1×10^{-1}
L47P	CUU to CCU	43.1±1.9	no	$< 1 \times 10^{-7}$	R119Q	CGA to CAA	30.2±1.2	yes	6×10^{-4}
R48C	CGU to UGU	35.8±1.9	yes	2×10^{-7}	F120S	UUU to UCU	36.1±2.0	yes	$< 1 \times 10^{-7}$

Q53L	CAG to CUG	39.3±2.7	yes	1×10^{-6}	F120L	UUU to CUU	18.1±0.9	yes	2×10^{-3}
N62Y	AAU to UAU	25.1±1.8	yes	3×10^{-3}	F121S	UUU to UCU	31.0±1.0	yes	$< 1 \times 10^{-7}$
Q63L	CAG to CUG	20.0 ±0.3	yes	3×10^{-2}	F121V	UUU to GUU	32.2±1.7	yes	$< 1 \times 10^{-7}$
N67T	AAU to ACU	38.1±1.2	yes	5×10^{-5}	F121L	UUU to CUU	31.9±0.7	yes	$< 1 \times 10^{-7}$
N67S	AAU to AGU	33.6±2.1	yes	1×10^{-4}	F121I	UUU to AUU	33.0±2.1	yes	$< 1 \times 10^{-7}$
K68E	AAA to GAA	35.5±1.0	yes	5×10^{-3}	F121C	UUU to UGU	31.2±1.6	yes	$< 1 \times 10^{-7}$

[a] The mutants are named after their codon changes. For example, N5D refers to the fifth amino acid (aspartamine, N) from the N- terminus of TraM replaced by an aspartic acid (D). A "*" represents a stop codon.

[b] Determined by assaying the β-galactosidase activity of cells containing pACPM24fs::*lacZ* and a pRFM200 derivative. Cells containing pACPM24fs::*lacZ* and pRFM200 had a β-galactosidase activity at 8 MU. Cells containing pACPM24fs::*lacZ* and pRFM200-Mdel (a pRFM200 derivative with *traM* deleted) had a β-galactosidase activity at 42 MU.

[c] Determined by immunoblot analysis (with anti-TraM antiserum) of 0.1 OD_{600} cells containing a pRFM200 derivative. Immunoblot bands at comparable intensity as that of 0.1 OD_{600} cells containing pRFM200 are considered "yes"; barely detected bands are considered "yes (-)"; no detectable bands are considered "no".

[d] Determined by assaying donor ability of cells containing pOX38-MK3 (TraM$^-$) and a pRFM200 derivative (containing a mutated *traM*). Cells containing pOX38-MK3 and a *traM*-deleted pRFM200 derivative (pRFM200-Mdel) did not have detectable donor ability, while the donor ability of the host cells containing pOX38-MK3 and pRFM200 (wild type *traM*) is 4×10^{-1}. No detectable donor ability is referred as "$<1 \times 10^{-7}$".

Chapter 5

Correlating Domains of TraM for Oligomerization and DNA Binding with Its Role in Autoregulation and F Conjugation

5.1 Introduction

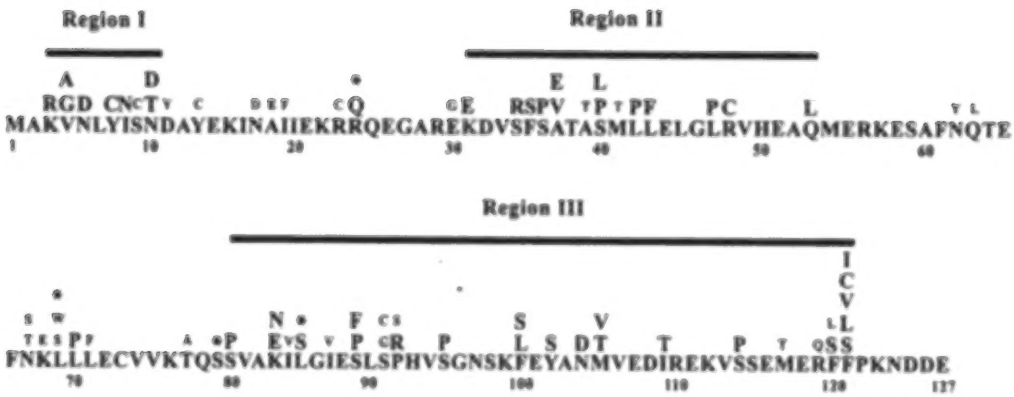
F plasmid *traM* encodes a 127-amino acid protein that is essential for conjugation (Kingsman and Willetts, 1978). TraM cooperatively binds to three cognate sites (*sbmA*, -*B*, -*C*) near *oriT* in the F plasmid (Di Laurenzio *et al.*, 1992; Fekete and Frost, 2000; Fekete and Frost, 2002). The sites with the highest affinity (*sbmA*) and the second highest affinity (*sbmB*) to TraM overlap the two *traM* promoters, allowing autoregulation of *traM* transcription (Di Laurenzio *et al.*, 1992; Penfold *et al.*, 1996; Fekete and Frost, 2002). The lowest affinity site, *sbmC*, is located nearest to *oriT*. Removal of *sbmA* and *sbmB* from an *oriT* fragment cloned in a multi-copy plasmid decreases conjugative transfer by 100-fold, whereas removal of *sbmC* further decreases transfer by 100-fold (Fu *et al.*, 1991).

TraM forms tetramers in solution with the oligomerization equilibrium between tetramers and monomers being two-state, suggesting that TraM tetramerization is so efficient that no other folding intermediates are detectable (Miller and Schildbach, 2003). This result is consistent with the report that R1 TraM binds to DNA as a tetramer (Verdino *et al.*, 1999), but argues against a model in which TraM initially binds to DNA as a dimer (Fekete and Frost, 2002). Deletion analysis has indicated that a TraM fragment of residues 2 to 55 forms dimers, whereas a TraM fragment with the C-terminal 70 (58-127) residues forms tetramers (Miller and Schildbach, 2003), suggesting that both regions are involved in TraM oligomerization.

The N-terminal region of TraM is important for cognate DNA binding and required for autoregulation and F conjugation (Schwab *et al.*, 1993; Miller and Schildbach, 2003); however, the role of the C-terminus remains ambiguous. Furthermore, the relevance of

TraM tetramerization to autoregulation and F conjugation as well as the structural domains that are responsible for the specificity and cooperativity of TraM in cognate DNA binding has not been investigated. A preliminary mutational analysis of TraM has shown that a missense mutation in either the C- or N-terminal region of TraM can affect TraM in both autoregulation and F conjugation, although the mechanisms behind these functional alterations are unknown (Chapter 4). In this work, TraM mutants were characterized by immunoreactivity, oligomerization, cognate DNA binding, and the activities in autoregulation and F conjugation to locate the functional domains of TraM. Results suggested that cognate DNA binding is essential for TraM in F conjugation, whereas oligomerization facilitates TraM binding to DNA.

Figure 5-1. Location of missense mutations in F plasmid TraM. Ordinal numbers are shown below the corresponding residues in the TraM amino acid sequence. The residue substitutions resulted from different missense mutations are indicated above the corresponding residues. If a TraM mutant complemented pOX38-MK3 transfer to a frequency at or higher than 10^{-5} transconjugants per donor (Table 4-2), the corresponding residue substitution was shown in smaller font size. Black boxes above the TraM sequence indicate the three regions (I, II, and III), which contain most of the missense mutations that decreases the ability of TraM to complement pOX38-MK3 transfer to a frequency lower than 10^{-5} transconjugants per donor.


Region I
Region II
Region III
MAKVNLYISNDAYEKINAIIEKRRQEGAREKDVSFSATASMLLELGLRVHEAQMERKESAFNQTE
FNKLLLECVVKTQSSVAKILGIESLSPHVSGNSKFEYANMVEDIREKVSSEMERFFPKNDDE

5.2 Results

5.2.1 Three regions of TraM important for F conjugation

A total of 72 missense mutations throughout *traM* have been collected, each of which affects TraM in both autoregulation and F conjugation (Chapter 4). All the mutants were previously cloned in pRFM200 derivatives, which constitutively express *traM* mutants from an attenuated P_{traJ} at low levels. The mutations that affected the TraM function for F conjugation most seriously were concentrated in regions I, II, and III of TraM, which corresponded to residues 3 to 10, 31 to 53, and 80 to 121, respectively (Figure 5-1).

In order to characterize *traM* mutants transcribed from their native promoter (P_{traM}), a region II mutant, K31E, and a region III mutant, I109T, were cloned downstream of P_{traM} in a medium-copy vector, pT7-4. Both pJLM103 (P_{traM}K31E) and pJLM104 (P_{traM}I109T) overexpressed mutants compared to pJLM102 (P_{traM}*traM*), whereas the growth rates of their host cells were not affected (data not shown), indicating that cells could tolerate TraM overexpression to a certain level. In cells containing pACPM24fs::*lacZ*, overexpressed I109T from pJLM104 exerted nearly the same level of repression of P_{traM} as wild type TraM (pJLM102). However, overexpression of I109T or K31E only resulted in low levels of complementation of pOX38-MK3, suggesting that F conjugation had more requirements for TraM than autoregulation. When co-resident with a wild-type F derivative, pOX38-Km, both mutants decreased the transfer frequency of pOX38-Km by more than 400 fold (Table 5-1), suggesting interactions or competitions between mutant and wild type TraM, which led to negative dominance over F conjugation.

Table 5-1. Properties of *traM* and its mutants when expressed from P_{traM}

Construct	Cloned gene	Expression of TraM or its mutants [a]	Regulating P_{traM} (MU) [b]	Complementing pOX38-MK3 [c]	Effect on pOX38-Km [d]
pJLM102	*traM*	regulated	5.3 ± 0.5	4×10^{-1}	8×10^{-1}
pJLM103	K31E	overexpressed	34.8 ± 1.5	6×10^{-5}	2×10^{-3}
pJLM104	I109T	overexpressed	7.9 ± 0.9	8×10^{-6}	1×10^{-3}
pT7-4	none	none	42.8 ± 2.1	$< 10^{-7}$	6×10^{-1}

[a] Determined by immunoblot analysis (with anti-TraM antiserum) of 0.1 OD_{600} of cells containing the corresponding construct.

[b] Determined by assaying the β-galactosidase activity of cells containing pACPM24fs::*lacZ* (with P_{traM} fused to the *lacZ* reporter gene) and a listed construct. Wild-type repression is represented by pJLM102. Complete loss of repression is represented by vector control, pT7-4.

[c] Determined by assaying the donor ability of cells containing pOX38-MK3 (a *traM*-deficient F derivative) and the corresponding construct.

[d] Determined by assaying the donor ability of cells containing pOX38-Km (a wild type F derivative) and the corresponding construct.

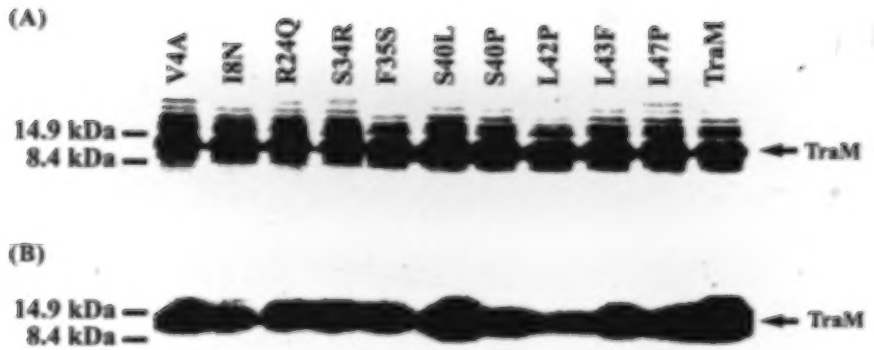
5.2.2 Some N-terminal mutations reduced TraM immunoreactivity

Some TraM mutants with residue substitutions in the N-terminal region were not detected by TraM antiserum in the previous analysis (Chapter 4), suggesting that these residue substitutions either increased protein instability or reduced protein immunoreactivity. To ensure against these unwanted phenotypes, these *traM* mutants were cloned into pBluescript KS+ to give pJLM400 derivatives such that TraM mutants were overexpressed and thereby detectable by Coomassie blue staining of an SDS-polyacrylamide gel (Chapter 3). Without glucose repression, pJLM400 derivatives overexpressed TraM mutants at equivalent levels as pJLM400 (wild type TraM) (Figure 5-2A). The immunoblot of an equivalent gel revealed that the mutants did not react with anti-TraM antiserum as efficiently as wild type TraM (Figure 5-2B). Thus, these tested N-terminal mutants had lower immunoreactivity to anti-TraM antiserum rather than having reduced protein stability. For unknown reasons, the nonsense mutants L69* and L85* were overexpressed as truncated TraM fragments at much lower levels than S79*, as were K3R and N10T in comparison to TraM (data not shown).

5.2.3 Analysis of TraM mutants using native gel electrophoresis

In order to determine how TraM was altered by different mutations, TraM and its mutants were analyzed by native polyacrylamide gels (Figure 5-3A). Mutants that had a residue substituted by an aspartate (D) or a glutamate (E), both of which are negatively charged, exhibited dominant species of greater mobility than TraM and the other mutants, indicating a gain of intrinsic negative charge for those mutants. These results agreed with the observation that N5D and N10D were eluted more easily (at lower salt concentration)

Figure 5-2. Overexpression of wild type and mutant TraM. Cells containing pJLM400 (*traM*) or its derivatives (*traM* mutants) were grown in LB broth for 3 hours before harvest. All the mutants are named after their mutations (Table 4-2). Samples were separated by 15% SDS-polyacrylamide gels. Locations of molecular weight markers are indicated on the left side of the figures. (A) TraM and its mutants detected by Coomassie blue staining. 0.1 OD_{600} of cells were loaded each lane. (B) TraM and its mutants detected by immunoblot with anti-TraM antiserum. 0.01 OD_{600} of cells were loaded each lane.


(A)
V4A
I8N
R24Q
S34R
F35S
S40L
S40P
L42P
L43F
L47P
TraM
14.9 kDa
8.4 kDa
TraM
(B)
14.9 kDa
8.4 kDa
TraM

than other mutants from a cation-exchange column (Materials and Methods).

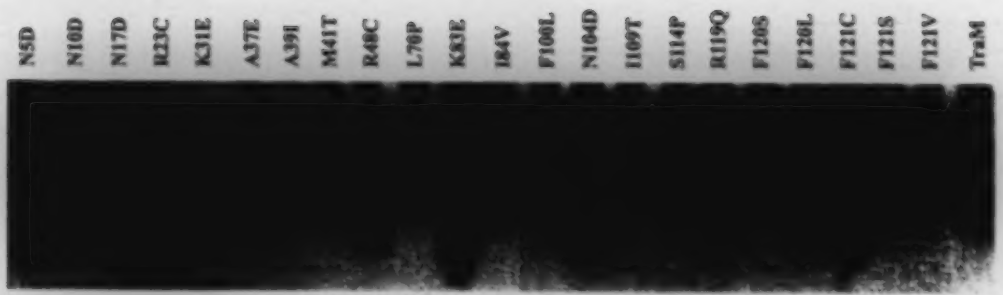
Because of such charge effects, the mobility of TraM mutants in the native polyacrylamide gel did not reflect their native sizes. Therefore, blue native gel electrophoresis was used to compare the native size of TraM mutants. The native gel samples were pre-bound with negatively charged Coomassie blue G-250 and run in buffer containing Coomassie blue G-250 so that the effect of the intrinsic charge on the electrophoretic mobility of native proteins was neglectable (Schagger and von Jagow, 1991). In a blue native gel, dominant species of TraM as detected by anti-TraM antiserum ran close to the 66-kDa BSA monomers, indicating that TraM formed tetramers (Figure 5-3B, top panel). Species bigger than TraM tetramers were also detected, indicating the existence of higher aggregates. Most mutants, especially C-terminal mutants, exhibited species smaller than tetramers. Analysis of the native gel samples with SDS-PAGE did not find degraded TraM mutants detectable by anti-TraM antiserum (Figure 5-3B, bottom panel), indicating that the smaller-than-tetramer species resulted from defective tetramerization of TraM mutants rather than protein degradation. Due to the resolution limit of blue native gel electrophoresis (Schagger and von Jagow, 1991; Schagger *et al.*, 1994), the observed smaller-than-tetramer species in TraM mutants could not be determined as dimers (approx. 29 kDa) or monomers (approx. 14.5 kDa).

5.2.4 Purification and oligomerization of TraM and its mutants

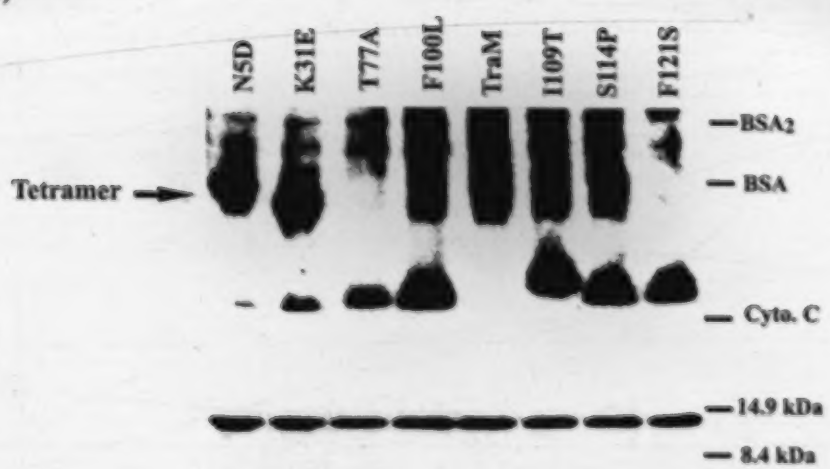
Oligomerization and cognate DNA binding are two major characteristics of TraM, which could be responsible for its autoregulation function (Di Laurenzio *et al.*, 1992; Verdino *et al.*, 1999; Miller and Schildbach, 2003). For further characterization, 20 TraM

Figure 5-3. Native polyacrylamide gel analysis of TraM and its mutants. TraM and its mutants were detected by immunoblot with anti-TraM antiserum. (A) TraM and TraM mutants separated by native polyacrylamide gel. (B) *Top panel*, TraM and its mutants separated by blue native polyacrylamide gels. Locations of protein standards including bovine serum albumin monomers (BSA; 66 kDa) and dimers (BSA_2), and cytochrome C (Cyto.C; 12 kDa) are indicated on the right side of the figure. *Bottom panel*, the native gel samples separated by 15% SDS-polyacrylamide gels. Protein weight markers are indicated on the right side of the figure.

(A)


N5D
N16D
N17D
R23C
K31E
A37E
A39I
M41T
R48C
L70P
K83E
I84V
F100L
N104D
I109T
S114P
R119Q
F120S
F120L
F121C
F121S
F121V
TraM

(B)

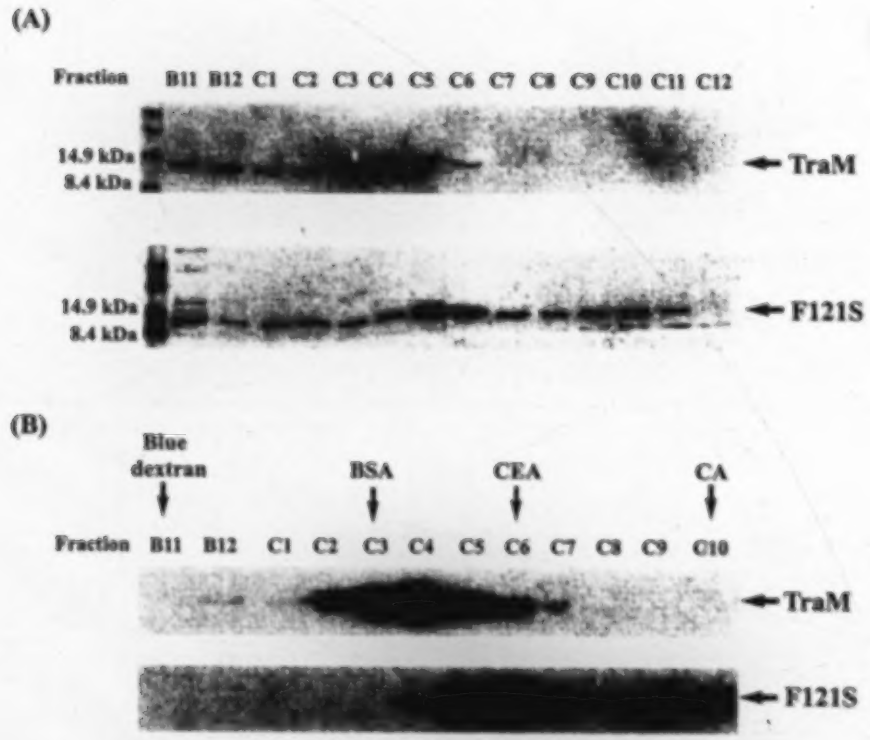

N5D
K31E
T77A
F100L
TraM
I109T
S114P
F121S
Tetramer
BSA2
BSA
Cyto. C
14.9 kDa
8.4 kDa

mutants with single residue substitutions in regions I, II, or III were selected for purification. TraM and twelve mutants were successfully purified (Materials and Methods), whereas R24Q, K31E, L42P, L47P, S89F, S95P, F100S, and S114P did not bind to the cation exchange column (MonoS HR 5/5) efficiently and thus were not purified. During preparative size exclusion chromatography, TraM and its missense mutants had a pure major peak and a minor peak at B11 with contaminants of higher molecular weight (Figure 5-4A). For F121S as well as I109T, F120S, F121V, a second minor peak appeared at C10 contained degraded proteins detectable by anti-TraM antiserum (data not shown).

Because of the contaminants in minor peaks, it was not clear whether those minor peaks resulted from altered oligomerization or from aggregation with other cellular proteins. In order to determine the oligomerization status of these mutants, the native sizes of pure TraM and its mutants were estimated using analytical size exclusion chromatography (Materials and Methods). TraM came out as a single peak at C4 (Figure 5-4B), which corresponded to the size of a tetramer (approx. 58 kDa), whereas the peak of higher aggregates (B11) observed in preparative SEC was barely detectable. Similarly, the B11 peak of F121S in preparative SEC was not detected in analytical SEC either (Figure 5-4B). These results indicated that the higher aggregates of TraM and its mutants detected in preparative SEC mainly resulted from aggregation of TraM and contaminants.

The elution profile of TraM mutants in analytical SEC fell into four groups. In the first group, F121S had a major peak at C6 corresponding to a size of 45 kDa (Figure 5-4B). Because the shape of a protein can affect its mobility in a size exclusion column (Hagel, 1998) and because F121S had a minor peak (C9) corresponding to the size of

Figure 5-4 Preparation and analysis of TraM and its mutants using size exclusion chromatography. Samples were separated by 15% SDS-polyacrylamide gels. Fraction numbers are marked above each lane. Peaks of different protein standards are shown above the figures. Blue dextran is an indicator of the column void volume (2000 kDa); BSA represents bovine serum albumin monomer (66 kDa); CEA stands for chicken egg albumin (45 kDa); CA stands for carbonic anhydrase (29 kDa). (A) Preparative SEC fractions of TraM and F121S as detected by Coomassie blue staining. The first lane on the left of each figure was loaded with protein weight markers. (B) Analytical SEC fractions of TraM and F121S as detected by immunoblot with anti-TraM antiserum. (C) Analytical SEC fractions of purified N5D and A37V as detected by immunoblot with anti-TraM antiserum. (D) Analytical SEC fractions of purified S79* as detected by immunoblot with anti-TraM antiserum.


(A)
Fraction B11 B12 C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11 C12
14.9 kDa
8.4 kDa
TraM
14.9 kDa
8.4 kDa
F121S
(B)
Blue dextran
BSA
CEA
CA
Fraction B11 B12 C1 C2 C3 C4 C5 C6 C7 C8 C9 C10
TraM
F121S
(C)
BSA
CEA
CA
Fraction C2 C3 C4 C5 C6 C7 C8 C9 C10
N5D
A37V
(D)
BSA
CEA
CA
Fraction C3 C4 C5 C6 C7 C8 C9 C10 C11 C12 D1 D2 D3 D4 D5
S79*

dimers (29 kDa), the major peak of F121S was designated as tetramers that were different from TraM tetramers in conformation. I109T, F120S and F121V shared identical elution profiles with F121S (data not shown). In the second group, N5D (Figure 5-4C) as well as V4A, N10D, and F120L (data not shown) had identical elution profiles as wild type TraM, indicating normal tetramerization for these mutants. In the third group, A37V (Figure 5-4C) as well as R48C and Q53L (data not shown) had a major peak identical to that of TraM tetramers and a minor peak at C7, which was designated as dimers that were different from the dimers of F121S in conformation. In the fourth group, S79*, a truncated TraM fragment missing the C-terminal 49 residues, had a major peak at D2 corresponding to the size of 18-kDa dimers and a minor peak at C6 close to the size of 36-kDa tetramers (Figure 5-4D).

These results indicated that the smaller-than-tetramer species observed in blue native gel electrophoresis were dimers. Defective tetramerization of TraM mutants correlated with their defective functions for autoregulation and F conjugation, suggesting that TraM tetramerization is important for its functions.

5.2.5 Cognate DNA binding of TraM and its mutants

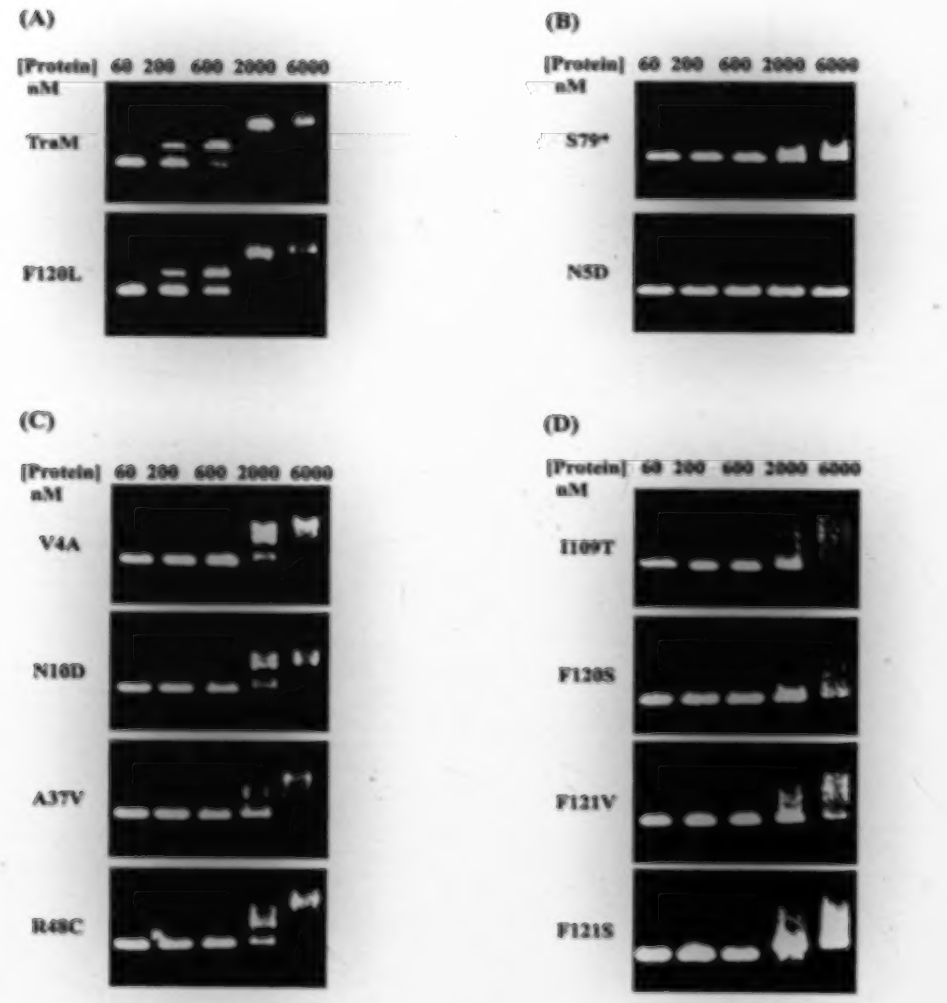
In order to determine the DNA binding ability of TraM mutants, electrophoretic mobility shift assays (EMSA) were performed and categorized TraM and its mutants into four groups according to their patterns of binding to *sbmABC*, which contains the three TraM binding sites. In the first group (Figure 5-5A), TraM bound to *sbmABC* with high affinity. At 600 nM of TraM (equal to 150 nM of TraM tetramers), 40 nM of *sbmABC* was almost completely shifted. Within a 3-fold increase of protein concentration, the

shifted complex was shifted to a higher order. As shown by Fekete and Frost (2002), the first-order shift of *sbmABC* was caused by TraM bound at *sbmA*, and the second-order shift was caused by TraM bound at both *sbmA* and *sbmB*, an indication of differentiated affinity of TraM to its cognate sites. In the same group, F120L bound to *sbmABC* with slightly lower affinity than TraM (Figure 5-5A).

In the second group (Figure 5-5B), N5D and S79* exhibited no significant shift of *sbmABC* at the highest tested concentration (6000 nM), indicating a loss of DNA binding ability for these two mutants. In the third group (Figure 5-5C), V4A, N10D, A37V, and R48C needed 3 to 10 fold higher concentrations than TraM to completely shift *sbmABC*, indicating decreased DNA binding affinity for these mutants. In the fourth group (Figure 5-5D), I109T, F120S, F121V, and F121S, which had similarly lowered binding affinity to *sbmABC* as the third group mutants, shifted *sbmABC* into smears, which represented a mix of multiple binding complexes of different electrophoretic mobility. As controls, I109T and F121V shifted *sbmA* into a uniform complex rather than a smear (Figure 5-5E). Therefore, the formation of multiple *sbmABC* binding complexes (a smear) appeared to be caused by a mutated protein bound to different sites in *sbmABC* since the position of a bound protein affects the electrophoretic mobility of the binding complex (Lane *et al.*, 1992; Fekete and Frost, 2002)

Therefore, regions I, II, and III (Figure 5-1) all appeared to be important for the high binding affinity of TraM to its cognate sites, whereas region III was also involved in maintaining the differentiated affinity that allowed TraM to bind in order to *sbmA*, *-B*, *-C*. All the tested mutants were defective in cognate DNA binding, suggesting that TraM binding to *oriT* is essential for both autoregulation and F conjugation.

Figure 5-5. DNA Binding of TraM and its mutants as determined using EMSA. Increased concentrations of TraM or its mutants in the reactions were indicated above each figure. Each reaction used 40 nM of DNA fragments containing all three TraM binding sites (*sbmABC*) or a single site (*sbmA*). (A) Binding of TraM or F120L to *sbmABC*. (B) Binding of S79* or N5D to *sbmABC*. (C) Binding of V4A, N10D, A37V, or R48C to *sbmABC*. (D) Binding of I109T, F120S, F121V or F121S to *sbmABC*. (E) Binding of TraM, I109T, or F121V to *sbmA*.


(A)
[Protein] 60 200 600 2000 6000
nM
TraM
F120L
(B)
[Protein] 60 200 600 2000 6000
nM
S79*
N5D
(C)
[Protein] 60 200 600 2000 6000
nM
V4A
N10D
A37V
R48C
(D)
[Protein] 60 200 600 2000 6000
nM
I109T
F120S
F121V
F121S
(E)
[Protein] 60 200 600 2000 6000 12000
(nM)
TraM
[Protein] 60 200 600 2000 6000 12000
(nM)
I109T
[Protein] 60 200 600 2000 6000 12000
(nM)
F121V

5.3 Discussion

Regions I (residues 3 to 10), II (residues 31 to 53), and III (residues 80 to 121) of TraM are required for F conjugation (Figure 5-1). All the tested TraM mutants with residue substitutions in these regions were defective in binding to *sbmABC*, suggesting that cognate DNA binding is required for TraM to function in autoregulation and F conjugation. However, the three regions of TraM appeared to contribute to cognate DNA binding through different mechanisms.

Mutations within region I did not affect TraM tetramerization but decreased the binding affinity of TraM to *sbmABC*, suggesting that this region directly participate in DNA binding. Asparagine (N) is the third most common residue to form hydrogen bonds with bases and DNA backbones in protein-DNA complexes (Luscombe *et al.*, 2001). The residue 5 (N) might interact with the negatively-charged DNA; thus replacement of this residue with a negatively-charged residue (aspartic acid, D) abolished the ability of TraM to bind to *sbmABC* (Figure 5-5B). When residue 10 (N) of TraM was replaced by the 10^{th} residue (D) in R1 TraM (Finlay *et al.*, 1986a), the resulting mutant protein bound to *sbmABC* less efficiently (Figure 5-5C), suggesting that residue 10 contributed to the allelic specificity of F-like TraM during DNA binding (Fekete and Frost, 2000). This agreed with a previous finding that the first 24 residues of R1 TraM define the DNA binding specificity (Schwab *et al.*, 1993).

Mutations within region II affected TraM in tetramerization. A37V, R48C, and Q53L formed tetramers and a smaller number of dimers. The two monomers in the dimer could be attached at the C-terminus but separated at the N-terminus, resulting in a larger apparent size than expected when determined by analytical size exclusion

chromatography (Figure 5-4C; Hagel, 1998). Since a TraM fragment of residues 2 to 55 forms dimers, region II could be responsible for the N-terminal dimerization that appeared to facilitate tetramerization of the full-length TraM, although the C-terminal region of TraM is able to form tetramers alone (Miller and Schildbach, 2003). Because A37, R48, and Q53 were involved in interactions between TraM monomers, they were unlikely to be present at the surface of the TraM tetramer to interact with DNA. Therefore, the decreased affinity of A37V, R48C (Figure 5-5C) and Q53L (data not shown) in cognate DNA binding appeared to result from the defective tetramerization of these mutants.

Missense mutations within region III appeared to affect the structure of TraM more dramatically. S89F, S95P, F100S, and S114P did not bind to the MonoS column efficiently under the conditions used in this work, suggesting that some major structural changes occurred in these mutant proteins. I109T, F120S, F121V, and F121S appeared to form conformationally altered tetramers and a smaller number of dimers as determined by analytical size exclusion chromatography (Figure 5-4B). F120L, which tetramerized efficiently and formed normal tetramers as TraM, bound to *sbmABC* in an identical pattern as TraM except with slightly lower affinity. Therefore, defective tetramerization and conformational changes of tetramers must be the reasons that I109T, F120S, F121V, and F121S bound to cognate sites with lower affinity and specificity than TraM (Figure 5-5D).

It could be argued that the major peaks of F121S (or other similar mutants) contained trimers or dimers with a bigger apparent size rather than tetramers; however, other evidence supported the interpretation that those major peaks contained conformationally

altered tetramers. Firstly, since the N-terminal region of TraM exists as dimers rather than monomers (Miller and Schildbach, 2003), oligomerization of the TraM mutant proteins that have an intact N-terminal region is unlikely to result in trimers. Secondly, since S79*, which does not have the C-terminal 49 residues of TraM, had a minor peak of tetramers (Figure 5-4D), F121S, which only has one residue substituted at the C-terminal end of TraM should have a peak of tetramers. Thirdly, I109T, F120S, F121V, and F121S were eluted from a cation-exchange column at slightly lower salt concentration than TraM (Materials and Methods), suggesting a conformational change for these mutants.

Blue native polyacrylamide gel electrophoresis determined that I109T and F121S had more dimers than tetramers (Figure 5-3B), which was opposite to the results obtained by analytical SEC (Figure 5-4B). Native gel samples were prepared from cells expressing levels of wild type or mutant TraM comparable to the physiological levels of TraM expressed by the F plasmid (Chapter 4), suggesting that I109T and F121S form more dimers than tetramers under physiological conditions. Although analytical SEC is comparatively accurate in estimating native sizes of protein complexes because the potential charge effects are ruled out, TraM mutants used in analytical SEC were more concentrated than in native gel electrophoresis and thus might tend to aggregate to form more tetramers than dimers.

Mutations that decreased TraM immunoreactivity are located within the first 47 residues of TraM (Table 4-2; Figure 5-2). Since prolines disrupt protein higher structure, mutations such as L42P and L47P could have altered the structure of TraM, hiding residues normally interacting with TraM antibodies, which agreed with the observation that L42P and L47P did not bind to MonoS column as efficiently as the mutants that were

purified. Other mutations could have changed the residues interacting with TraM antibodies, resulting in reduced immunoreactivity of corresponding mutants. The residues interacting with TraM antibodies must be at the surface of TraM tetramers, and thus might also directly interact with DNA during TraM binding at *oriT*. Most mutations that affected TraM immunoreactivity were highly defective for autoregulation and F conjugation (Figure 5-1; Figure 5-2), probably because the mutations also affected TraM activity in DNA binding as happened to V4A (Figure 5-5C).

Nearly normal repression for P_{traM} was observed when I109T was overexpressed from P_{traM}, indicating that increased dosage of I109T compensated for the defect of I109T in autoregulation. This agreed with our previous argument that expressing mutants of an autoregulatory gene from its native promoter compromises functional characterization because of the compensation by protein overexpression (Chapter 4). The result also suggested that a physical blockage was sufficient for repressing P_{traM}, despite that I109T was defective for tetramerization. However, overexpression of I109T from P_{traM} did not complement pOX38-MK3 very well in conjugative DNA transfer, indicating that F conjugation requires more stringent structural integrity of TraM than autoregulation although both functions of TraM shared extensive structural determinants.

In conclusion, this work has defined three domains of TraM involved in tetramerization or DNA binding, the two properties important for TraM to function in autoregulation and F conjugation. The N-terminal region of TraM (region I) is required for DNA binding and defines its allelic specificity, agreeing with previous results that the N-terminus of TraM from F-like R1 is responsible for specific DNA binding (Schwab *et al.*, 1993; Kupelwieser *et al.*, 1998). Tetramerization of F plasmid TraM increases the

binding affinity of TraM to its cognate sites, consistent with the finding that R1TraM binds to DNA as a tetramer (Verdino *et al.*, 1999). Involvement of extensive regions (II and III) in oligomerization presumably ensures efficient tetramerization of TraM, which could explain why dimers are not detectable between tetramers and monomers in the equilibrium studies of Miller and Schildbach (2003). The C-terminal domain (region III) of TraM is not only involved in tetramerization but also appears to define the different affinities of TraM for its three cognate sites, which could be related to the cooperativity of TraM in DNA binding (Fekete and Frost, 2002).

I propose that TraM forms a DNA binding domain at the N-terminus (region I) through tetramerization at regions II and III. Through cooperative binding, TraM occupies all three sites at *oriT* during relaxosome formation. The F relaxosome normally resides at the cell center or quarter position (Niki and Hiraga, 1997), and there is no DNA transfer or conjugative DNA synthesis (Kingsman and Willetts, 1978). After donor and recipient cells contact each other, TraM might connect the relaxosome to the transferosome through interactions with TraD (Disque-Kochem and Dreiseikelmann, 1997). TraM-TraD interactions might further change the binding properties of TraM at *oriT*, causing localized denaturation between *sbmABC* and the *nic* site. This localized melting at *oriT* could provide a region of single-stranded DNA that is required for inducing DNA helicase activity of TraI to further unwind the DNA for conjugative transfer (Csitkovits and Zechner, 2003). In this manner, TraM might indeed act as a "signaling factor" to trigger conjugative DNA transfer as proposed by Willetts and Wilkins (1984).

Chapter 6

F Conjugation Requires Interactions between TraD and the C-terminal Region of TraM

6.1 Introduction

Conjugation is one of the major mechanisms by which bacteria acquire new genes to exploit new environments and respond to selective pressure. Formation of a cytoplasmic nucleoprotein complex called the relaxosome and a transmembrane multi-protein complex called the transferosome is required for conjugative transfer of plasmids. During conjugation, specific interactions between relaxosome and transferosome might occur, allowing a single strand of plasmid DNA to be transferred from a donor cell into a recipient through the mating pore.

The F plasmid is the paradigm for a large group of conjugative plasmids in the IncF incompatibility complex that carry genes important for human and veterinary medicine, such as antibiotic resistance and toxin production. F plasmid TraM is essential for F conjugation but is not required for F-pilus assembly or mating pair formation (Willetts and Wilkins, 1984). TraM mainly exists in the cytoplasm whereas a smaller amount has also been found in the inner membrane (Di Laurenzio *et al.*, 1992). TraM forms tetramers and cooperatively binds to three sites (*sbmA*, *-B*, *-C*) at *oriT* in the F plasmid (Di Laurenzio *et al.*, 1992; Fekete and Frost, 2002; Miller and Schildbach, 2003). However, TraM is not important for the nicking reaction catalyzed by TraI in the presence of IHF and TraY (Howard *et al.*, 1995; Nelson *et al.*, 1995; Frost and Fekete, 2000). *In vitro* assays have shown that TraM interacts with TraD, an inner membrane component of the transferosome (Disque-Kochem and Dreiseikelmann, 1997). These findings support the hypothesis that TraM performs a signaling function between the cytoplasmic relaxosome and the membrane-bound transferosome during F conjugation (Willetts and Wilkins, 1984).

F plasmid TraD is an inner membrane protein with two membrane spanning regions such that the amino- and carboxyl-terminal regions are in the cytoplasm (Lee *et al.*, 1999). TraD is essential for conjugation but functions after mating pair formation and DNA processing (Everett and Willetts, 1980; Kingsman and Willetts, 1978; Panicker and Minkley, 1985). TraD belongs to the "TraG" family of coupling proteins that includes RP4 TraG, R388 TrwB, and VirD4 in the *Agrobacterium tumefaciens* T-DNA transfer system, which couple the relaxosome to the transport site during conjugation (Cabezón, *et al.*, 1997). Disque-Kochem and Dreiseikelmann (1997) who demonstrated that F TraM interacts with F TraD *in vitro* first suggested specific interactions between TraG family proteins and the relaxosome. TraG family proteins not only interacts with the relaxosome but also interacts with other transferosome components (Szpirer *et al.*, 2000; Schroder *et al.*, 2002; Llosa *et al.*, 2003; Gilmour *et al.*, 2003), further suggesting that coupling proteins connect the relaxosome to the transferosome. Coupling proteins might also serve as a pump to push the single stranded DNA through the mating pore (Gomis-Ruth *et al.*, 2001; Llosa *et al.*, 2002).

There has been no direct evidence that TraM-TraD interactions are required for F conjugation; nor are the regions of TraM and TraD involved in these interactions known. By screening for TraM mutants that were competent in DNA binding and tetramerization but highly defective for F conjugation, I selected a mutant, K99E, that was defective in interactions with TraD as determined by co-immunoprecipitation and affinity chromatography. The results suggest that interactions between TraD and the C-terminal region of TraM are required for F conjugation and that the residue K99 of TraM is important for interactions with TraD.

Table 6-1. Properties of TraM and its mutants [a]

TraM or its mutant	Codon change	Detection of TraM [b]	Regulating P_{traM}, (MU) [c]	Complementing pOX38-MK3 [d]	Oligomer-ization [e]	Binding to *sbmABC* [f]
TraM	none	yes	8.3 ± 0.3	5×10^{-1}	tetramer	wild-type
Q78H	CAA to CAU	yes	10.3± 1.6	1×10^{-3}	tetramer	wild-type
K99E	AAA to GAG	yes	8.1 ± 0.2	$< 1 \times 10^{-7}$	tetramer	wild-type
V106A	GUU to GCU	yes	8.9 ± 0.7	5×10^{-5}	tetramer	wild-type
Mdel	*traM* deletion	no	43.5± 1.7	$< 1 \times 10^{-7}$	N. A.	N. A.

[a] TraM or TraM mutants were expressed by pRFM200 or pRFM200 derivatives, respectively. The mutants are named after their codon changes as in the previous chapters.

[b] Determined by immunoblot analysis of 0.1 OD_{600} cells containing a pRFM200 derivative. Immunoblot bands at comparable intensity as that of 0.1 OD_{600} cells containing pRFM200 are considered "yes"; barely detected bands are considered "yes/no"; no detectable bands are considered "no".

[c] Determined by assaying the β-galactosidase activity of XK1200 cells containing pACPM24fs::*lacZ* (P_{traM} fused to the *lacZ* reporter gene), pOX38-MK3, and a corresponding pRFM200 derivative. Complete loss of autoregulation, represented by cells containing pACPM24::*lacZ*, pOX38-MK3, and a *traM* deleted pRFM200 derivative (pRFM200-Mdel).

[d] Determined by assaying donor ability of XK1200 cells containing pOX38-MK3, pACPM24::*lacZ*, and a pRFM200 derivative (containing a mutated *traM* gene). Cells containing pOX38-MK3 and a *traM*-deleted pRFM200 derivative (pRFM200-Mdel) did not have detectable donor ability (referred as $< 1 \times 10^{-7}$).

e Oligomerization was determined by analytical size exclusion chromatography as shown in Figure 6-1A. "N. A." refers to "not applicable".

[f] "Wild-type" indicates a binding ability to *sbmABC* identical to that of wild type TraM as determined by EMSA as shown in Figure 6-1B.

6.2 Results

6.2.1 Selection of autoregulation-competent TraM mutants that are defective for F conjugation

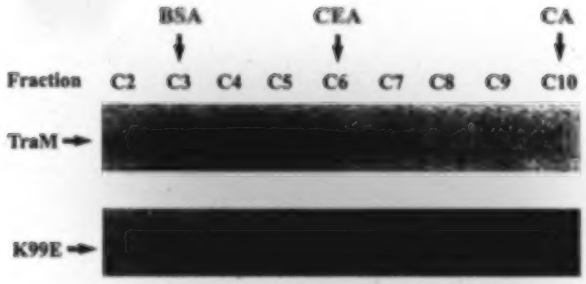
Our previous results suggested that both F conjugation and *traM* autoregulation involve extensive regions of TraM in oligomerization and cognate DNA binding (chapter 5). In order to determine whether or not there is a site in TraM that is exclusively involved in F conjugation, a three-plasmid system was used to select TraM mutants that were normal for autoregulation but defective for F conjugation.

In order to search sites throughout TraM, random PCR mutagenesis was used to amplify *traM* from pRFM200 (Materials and Methods). The mutated fragments were cloned into pT7-5 to give pRFM200 derivatives and were transformed into cells containing pOX38-MK3 (a *traM*-deficient F derivative) and pJLPM24fs::*lacZ* (P_{traM} fused to the *lacZ* reporter gene). Approximately 1% of transformants formed dark blue colonies on LB-plates containing X-gal, whereas the rest formed light blue colonies that expressed TraM (or its mutants) capable of repressing P_{traM} in pJLPM24::*lacZ*. Approximately 6,000 light blue colonies were patch-mated with fresh ED24 cells on plates containing Km and Spc (Materials and Methods). Three of these colonies did not produce transconjugants, suggesting that they were defective in conjugation but not autoregulation.

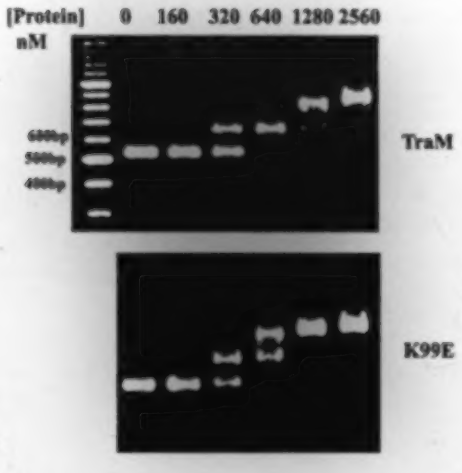
Plasmid DNA from these three colonies was isolated; and sequencing analysis found three missense mutations in *traM* including Q78H, K99E, and V106A (Table 6-1). TraM and the three mutants were at similar intracellular levels as determined by immunoblot

Figure 6-1. Analysis of tetramerization and DNA binding of TraM and K99E. (A) Analytical SEC fractions of purified TraM and K99E. 10 µl from each fraction was separated by 15% SDS-polyacrylamide gels and visualized by immunoblot with anti-TraM antiserum. Fraction numbers are indicated above each lane. The positions of different marker proteins are shown above the figure. BSA represents bovine serum albumin monomer (66 kDa); CEA represents chicken egg albumin (45 kDa); CA represents carbonic anhydrase (29 kDa). (B) Binding of TraM or K99E to *sbmABC*. Increasing concentrations of TraM or K99E (in nM) are shown above the figures. Each reaction contained 40 nM of *sbmA*. DNA molecular weight ladders are shown on the left of the figure.

(A)


BSA
CEA
CA
Fraction
C2
C3
C4
C5
C6
C7
C8
C9
C10
TraM
K99E

(B)


[Protein]
nM
0
160
320
640
1280
2560
600bp
500bp
400bp
TraM
K99E

analysis with anti-TraM antiserum (Table 6-1), indicating that neither protein stability nor immnunoreactivity of these mutant proteins was affected. The three colonies expressed β-galactosidase at similar levels as that containing wild type *traM* (Table 6-1). Donor ability assays indicated that these mutants, especially K99E, did not complement pOX38-MK3 in conjugative transfer as well as wild type TraM (Table 6-1). Our results indicate that there are residues in TraM such as Q78, K99 and V106 that are only involved in F conjugation but not autoregulation.

6.2.2 F conjugation requires a property of TraM besides tetramerization and DNA binding

In order to determine whether or not the autoregulation-competent TraM mutants were defective in tetramerization or cognate DNA binding, analytical size exclusion chromatography and electrophoretic mobility shift assays (EMSA) were used to analyze purified TraM and its mutant derivatives. TraM, Q78H, K99E, and V106A had the same single peak at C4 in analytical SEC, which corresponded to the size of TraM tetramers (Figure 6-1 A; Table 6-1). EMSA showed that each mutant protein shifted *sbmABC* to two different positions and at concentrations similar to wild type TraM (Figure 6-1 B; Table 6-1). Our results indicated that Q78H, K99E, and V106A did not affect oligomerization and DNA binding, suggesting that they affect a different property of TraM, which is also required for F conjugation.

6.2.3 Affinity chromatography analysis of TraM (or its mutants) -TraD interactions

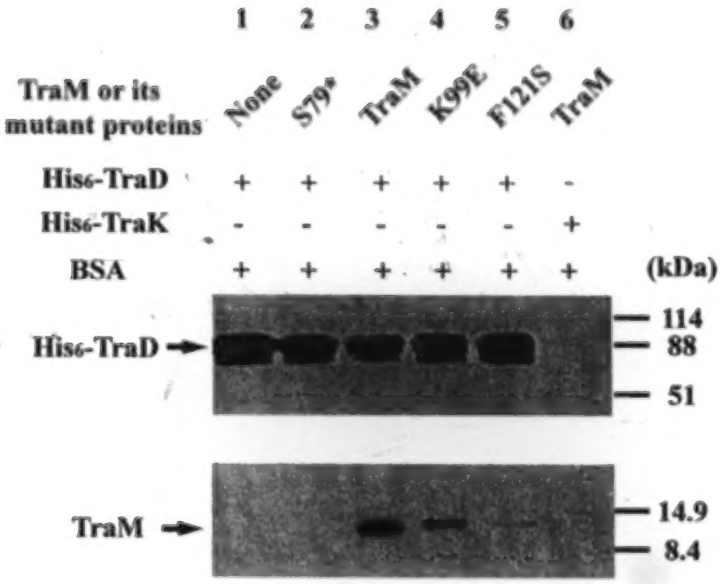
The ability to interact with TraD is another known property of TraM, and its potential importance for F conjugation has been suggested but is not yet proven (Disque-Kochem and Dreiseikelmann, 1997). Affinity chromatography was used to examine interactions between His_6-tagged TraD and TraM or K99E. S79*, a TraM mutant missing the C-terminal 49 residues, was also assayed to determine the importance of the C-terminal region of TraM for interactions with TraD (Chapter 5). F121S, a TraM mutant that forms dimers and conformationally altered tetramers, was also examined (Chapter 5).

TraM bound to His_6-TraD with the highest affinity among all the tested proteins, whereas His_6-tagged TraK, another transferosome component, did not appear to interact with TraM, suggesting specificity in TraM-TraD interactions (Figure 6-2 lane 3 and 6). K99E bound to His_6-TraD with significantly lower affinity than TraM (Figure 6-2 lane 4), whereas His_6-TraD-F121S interactions were even weaker (Figure 6-2 lane 5). S79* was not able to interact with His_6-TraD (Figure 6-2 lane 2). These results indicated that K99 of TraM is involved in TraM-TraD interactions that required the C-terminal region of TraM and that TraM tetramerization might also be important.

6.2.4 Co-immunoprecipitation analysis of TraM (or its mutants)-TraD interactions

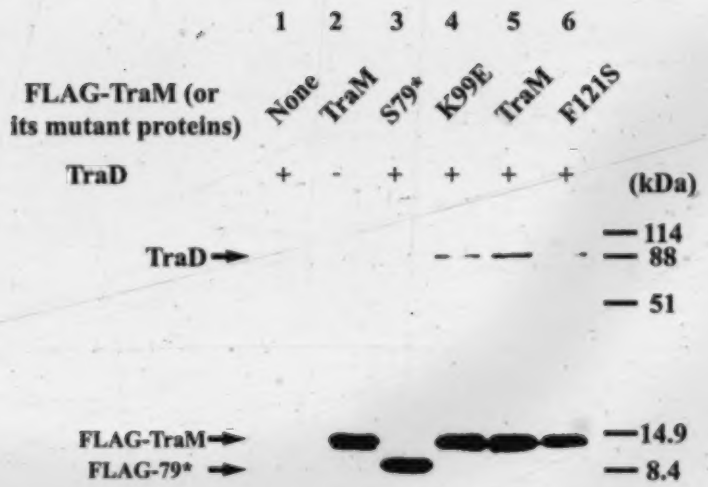
In order to verify results from affinity chromatography in which His_6-TraD was fixed to Ni-NTA agarose beads and used to capture TraM or its mutant proteins, co-immunoprecipitation assays were performed using M2 anti-FLAG agarose beads to precipitate FLAG-tagged TraM (or its mutant proteins) associated with TraD that was co-expressed.

Figure 6-2. His_6-TraD and TraM (or its mutant proteins) interactions as determined by affinity chromatography. Same amount of purified TraM or one of its mutant proteins was used to incubate with His-TraD (or His-TraK)-saturated Ni-NTA resin (Material and Methods). Immunoblot used anti-TraD (top panel) and anti-TraM antisera (bottom panel). Protein molecular weight makers are indicated on the right of the figure. Lane 1: His_6-TraD and bovine serum albumin; lane 2: plus S79*; lane 3: plus TraM; lane 4: plus K99E; lane 5: plus F121S; lane 6: His_6-TraK, bovine serum albumin, and TraM.


1 2 3 4 5 6
TraM or its mutant proteins
None
S79ᵃ
TraM
K99E
F121S
TraM
His6-TraD + + + + + -
His6-TraK - - - - - +
BSA + + + + + +
(kDa)
His6-TraD
114
88
51
TraM
14.9
8.4

From cells extracts containing both TraD and FLAG-TraM (or its mutants), M2 anti-FLAG agarose beads precipitated TraD together with FLAG-tagged TraM but not TraD alone (Figure 6-3 lane 1 and 5). FLAG-K99E and FLAG-F121S co-precipitated with less TraD (Figure 6-3 lane 4 and 6). FLAG-S79* was not unable to co-precipitate with TraD at all (Figure 6-2 lane 3). These results confirmed that K99E, F121S, and S79* had reduced affinity for TraD compared to TraM.

Figure 6-3. TraD and TraM (or one of its mutant proteins) interactions as determined by co-immunoprecipitation. Immunoblot used anti-TraD (top panel) and anti-TraM antisera (bottom panel). Protein molecular weight markers are indicated on the right of the figure. Each lane was loaded with 0.1 OD_{600} of cells expressing different proteins as indicated. FLAG-tagged TraM, S79*, K99E, and F121S were expressed by pJLM200, pJLFM201, pJLFM202, and pJLFM203, respectively. TraD was expressed by pJLD331. Lane 1: TraD; lane 2: FLAG-TraM; lane 3: TraD and FLAG-S79*; lane 4: TraD and FLAG-K99E; lane 5: TraD and FLAG-TraM; lane 6: TraD and FLAG-F121S


1 2 3 4 5 6
FLAG-TraM (or its mutant proteins)
None
TraM
S79*
K99E
TraM
F121S
TraD + - + + + +
(kDa)
114
88
51
TraD
FLAG-TraM
FLAG-79*
14.9
8.4

6.3 Discussion

TraM-TraD interactions have been previously shown with overlay assays and affinity chromatography (Disque-Kochem and Dreiseikelmann, 1997). Here we confirmed TraM-TraD interactions using affinity chromatography and co-immunoprecipitation using N-terminal His_6-tagged TraD and N-terminal FLAG-tagged TraM, respectively. The TraM mutant K99E, which was competent for autoregulation but not conjugation, was defective in interacting with TraD but competent in oligomerization and DNA binding to *sbmABC*. Deletion of the C-terminal region (S79*) or disruption of oligomerization (F121S) also affected TraM interactions with TraD. These results suggested that the TraM-TraD interaction is required for F conjugation and that the C-terminal region of TraM is involved in this process. K99E did not completely lose the ability to interact with TraD, suggesting that multiple residues within the C-terminal region of TraM could be involved in TraM-TraD interactions.

The C-terminal region of TraD corresponds to a cytoplasmic domain projecting from the inner membrane (Lee *et al.*, 1999). All the known F-like TraD proteins have a C-terminal domain that determines the specificity of plasmid mobilization (Chapter 8). A deletion of this domain in F TraD reduced specificity at the price of a lowered DNA transfer frequency (Sastre *et al.*, 1998). These results suggest that the C-terminal domain of TraD specifically recognizes the relaxosome, presumably through specific interactions with relaxosome components. Possible interactions between the C-terminal domains of TraM and TraD could explain our unsuccessful attempt to use the bacterial two-hybrid system for examining TraM-TraD interactions (data not shown). Because this *in vivo* protein interaction system requires tagging one target protein at the N-terminal end and

another at the C-terminal end, either way around, one of the tags could have blocked interactions between the proteins. TraM could also interact with domains of TraD besides that at the C-terminus since deletion of this domain reduced but did not abolish the function of TraD in F conjugation (Sastre *et al.*, 1998). R388 TrwA, which has been suggested to carry an activity similar to F TraM, interacts with TrwB (Llosa *et al.*, 2003), an equivalent of F TraD that lacks the C-terminal specificity domain (Chapter 8). This implies that interactions between TraM and TraD domains other than the specificity domain could be essential for F conjugation.

TraM-TraD interactions could be a key step to triggering conjugative transfer of F-like plasmids. The N-terminal domain of F plasmid TraM is heavily involved in interactions with F *oriT* (Chapter 5), leaving the free C-terminal domain to serve as an anchor to mediate specific interactions between the relaxosome and the transferosome. The relaxosome-transferosome interactions could cause topological changes on both sides of the complex in the inner membrane, triggering TraI to unwind DNA thereby detaching the transfer strand from the *nic* site via its helicase activity (Matson *et al.*, 2001). TraD might also serve as a DNA pump to further drive the transfer strand through the mating pore (Llosa *et al.*, 2002; Schroder *et al.*, 2002). However, the detailed functional domains of TraD have not been identified, leaving proposed functions of TraD mainly unproven.

Chapter 7

General Discussion

7.1 Two different functions of F plasmid *traM* (TraM)

F plasmid *traM* is the first gene downstream of *oriT* in the *tra* region and essential for F conjugation (Kingsman and Willetts, 1978; Frost *et al.*, 1994). Supplying TraM *in trans* does not fully complement *traM*-deficient F or F-like derivatives in conjugative transfer, especially when the host cells are recovering from stationary phase, suggesting an *in cis* function for *traM* in F conjugation (Chapter 3; Dempsey, 1994; Polzleitner *et al.*, 1997). P_{traM} could aid in this *in cis* function, since transcriptional readthrough from *traM* into *traJ* might link *traM* to a broader network of *tra* gene regulation (Polzleitner *et al.*, 1997; Stockwell and Dempsey, 1997; Chapter 3). Therefore, *traM* appears to participate in a regulatory circuit for *tra* gene expression.

TraM (14-kDa) forms tetramers thereby increasing its structural complexity and functional versatility (Chapter 5). TraM is a cytoplasmic protein essential for F conjugation and conjugative replication (Kingsman and Willetts, 1978; Di Laurenzio *et al.*, 1992). However, it is neither required for pilus assembly or mating pair formation (Achtman *et al.*, 1972; Achtman and Skurray, 1977, Achtman *et al.*, 1978) nor for nicking within *oriT*, a critical step in formation of the relaxosome (Everett and Willetts, 1980; Nelson *et al.*, 1995; Fekete and Frost, 2000). These properties of TraM support the hypothesis that TraM acts as a signaling factor to initiate DNA transfer in response to an unknown "mating signal" (Willetts and Wilkins, 1984). TraM binds to three sites at *oriT* and interacts with TraD in transferosome (Di Laurenzio *et al.*, 1992; Disque-Kochem and Dreiseikelmann, 1997; Fekete and Frost, 2002; Chapter 5; Chapter 6), implying that TraM physically connects the relaxosome to the transferosome in order to perform the "signaling" function.

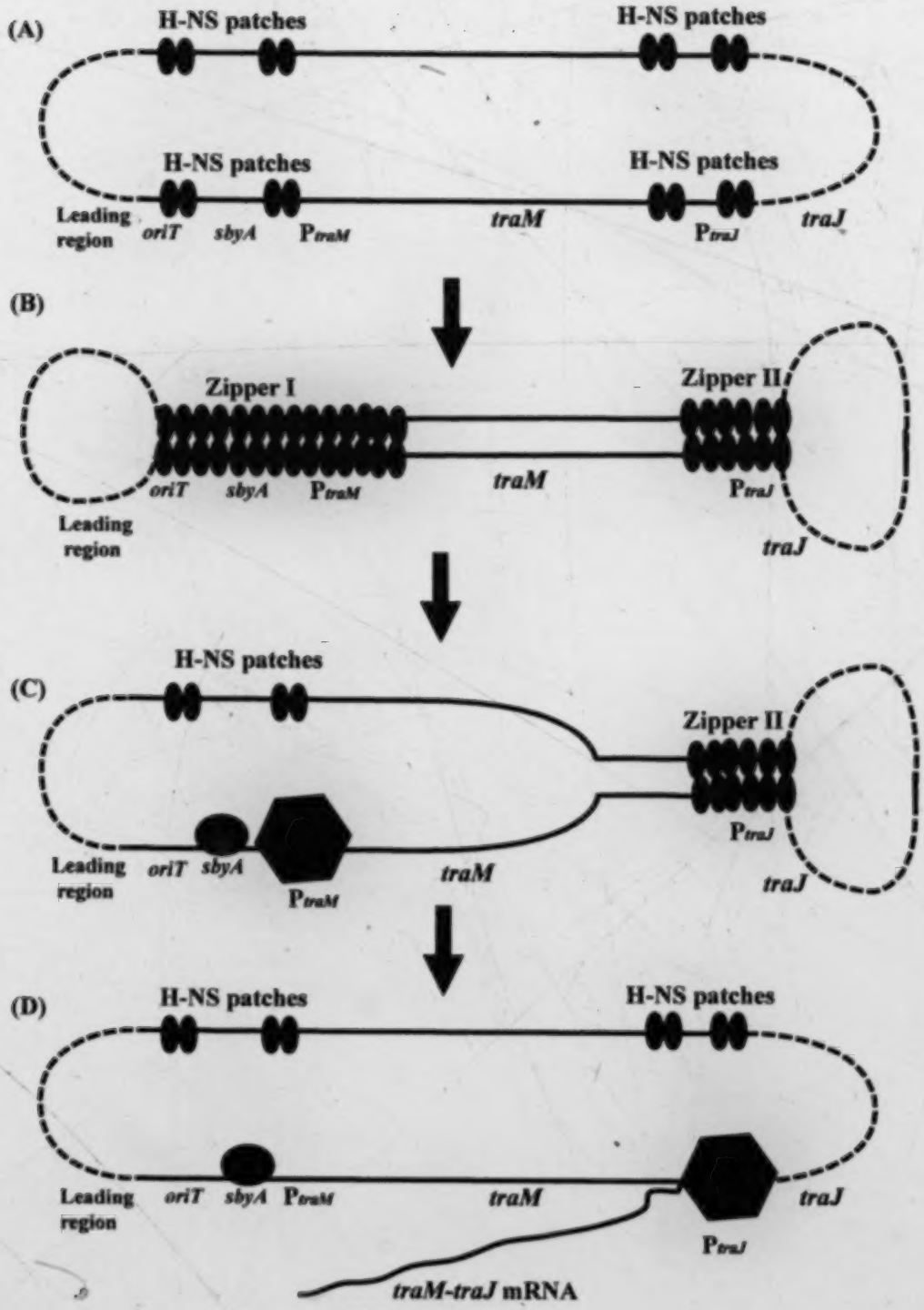
Taken together, results presented by previous studies and in this thesis suggest that *traM* contributes to F conjugation in two aspects, i.e. regulating *tra* gene expression and relaying the mating signal between the relaxosome and the transferosome. Detailed mechanisms related to these functions are further discussed in the following sections.

7.2 *traM* and *tra* gene regulation

The F plasmid appears to form a "closed" structure to repress *traM* and *traJ* expression in the absence of TraY (Chapter 3). Coincidentally, H-NS (heat-stable nucleoid structuring protein) silences *traM* and *traJ* only in the context of the F plasmid (R. Will, J. Lu, and L. S. Frost, unpublished data), implying that H-NS might participate in an *in cis* inhibition mechanism. In an *hns* strain, pOX38-Km continues to express *traM* and *traJ* in stationary phase and pOX38-*traY244* resumes TraM expression (R. Will and L. S. Frost, unpublished data), further suggesting that H-NS is involved in formation of the closed structure in the absence of TraY.

H-NS dimers bind to regions of curvature (Williams and Rimsky, 1997; Rimsky *et al*, 2001). There are H-NS binding sites upstream of P_{traM} and flanking P_{traJ} (Figure 7-1A; R. Will and L. S. Frost, unpublished data). Multiple patches of H-NS-decorated DNA could form zipper-like structures through aggregation of H-NS dimers on the DNA (Dorman and Deighan, 2003). Further aggregation of H-NS along the DNA results in zippers I and II, which prevent RNA polymerase from recognizing P_{traM} and P_{traJ}, respectively (Figure 7-1B). Zipper II might be less stable than zipper I, thus allowing low levels of *traJ* expression.

Figure 7-1. A model of the F plasmid resuming *tra* gene expression as the host cell recovers from stationary phase. (A) When host cells are at stationary phase and TraY is absent, H-NS dimers bind to regions of curvature adjacent to the TraY binding site (*sbyA*) and P_{traJ}. (B) H-NS patches aggregate to form zipper-like structures and further extend to block P_{traM} and P_{traJ}. Zipper II is shorter and thus less stable than zipper I, allowing low-level *traJ* transcription. (C) When host cells encounter agreeable growth conditions, the low level of *traJ* expression leads to low-level expression of TraY, which disrupts zipper I via binding to *sbyA* and allows RNA polymerase (RNAP) to load onto P_{traM} for transcription. (D) Strong transcription from P_{traM} extends into P_{traJ}; RNAP disrupts zipper II to fully de-repress P_{traJ}. High levels of TraJ fully activate the major *tra* operon, allowing the maximal transfer ability of the F plasmid.


(A)
H-NS patches
H-NS patches
H-NS patches
H-NS patches
Leading region
oriT
sbyA
PtraM
traM
PtraJ
traJ
(B)
Zipper I
Zipper II
oriT
sbyA
PtraM
traM
PtraJ
Leading region
traJ
(C)
H-NS patches
Zipper II
PtraJ
Leading region
oriT
sbyA
PtraM
traM
traJ
(D)
H-NS patches
H-NS patches
Leading region
oriT
sbyA
PtraM
traM
traJ
PtraJ
traM-traJ mRNA

In stationary phase, F^+ cells do not have detectable levels of TraM and TraY (Frost and Manchak, 1998; R. Will and Frost, unpublished data), the F plasmid forms the closed structure that represses *traM* and *traJ* transcription (Chapter 3). Changes in levels of host factors such as cAMP-CRP or LRP could be responsible for complete inhibition of TraJ expression (Camacho and Casadesus, 2002; Starcic *et al.*, 2003; Starcic-Erjavec *et al.*, 2003). Similarly, naturally transfer-repressed F-like plasmids do not express detectable TraJ, and thus the major *tra* operon including *traY* is not expressed. Without TraY, the plasmid forms the closed structure, resulting in complete inhibition of *traM*.

When cells encounter agreeable growth conditions, leaky expression of TraJ from the closed structure allows low-level expression of TraY, which binds to *sbyA* with high affinity (Lum *et al*, 2002) thereby disrupting zipper I and allowing RNA polymerase to bind to P_{traM} (Figure 7-1C). Transcription from P_{traM} not only efficiently increases levels of TraM but also disrupts the zipper II via readthrough into *traJ* by RNA polymerase (Figure 7-1D). Increased TraJ further increases expression of the major *tra* operon including *traY*. These positive feedback mechanisms enable the F plasmid to quickly regain maximal expression of conjugation-related proteins and efficiently transfer to F^- species. Similar mechanisms might apply to F-like plasmids in de-repressed expression of conjugation-related proteins before establishment of FinOP inhibition in new transconjugants. The temporary de-repression of transfer protein expression allows epidemic spread of the naturally transfer-repressed F-like plasmids from new transconjugants to recipients. After establishment of FinOP inhibition in a new transconjugant, cell division dilutes the concentrations of transfer proteins. Consequently,

transconjugants lose the abnormal donor ability after 2-7 generations (Stocker *et al.*, 1963; Monk and Clowes, 1964).

The F plasmid promotes recombination by forming Hfr strains and F-like plasmids carry various antibiotic resistance genes, which are beneficial for host cells. Although *tra* gene products are normally not essential for bacterial growth and increase the sensitivity of host cells to bacteriophages, FinOP inhibition and the *in cis* inhibition mechanism can combine to stringently control expression of *tra* genes. FinOP inhibition keeps *tra* gene expression at minimal levels except when host cells encounter an agreeable environment and F^- recipient cells, the epidemic spread mechanism enables plasmids to temporarily transfer at high frequencies and saturate the F^- cell population. On the other hand, the *in cis* inhibition mechanism not only extends FinOP inhibition to *traM* but also controls *tra* gene expression in $FinOP^-$ plasmids. When host cells are in limiting growth conditions, the F plasmid would form the closed structure to eventually shut down all *tra* gene expression, which is obviously helpful for the host cells.

In conclusion, *traM* appears to facilitate F and F-like plasmids in conjugative DNA transfer by up-regulating *traJ*, although the proposed closed structure of the F plasmid and its relation to H-NS and other factors require further study.

7.3 TraM and conjugative DNA transfer

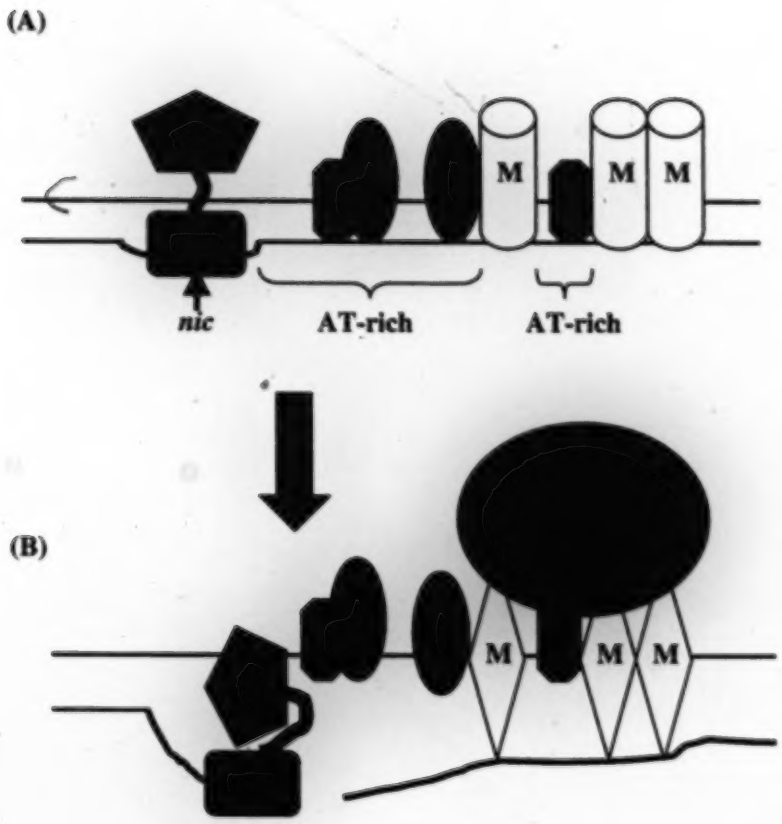
The F relaxosome is a double-stranded (ds) DNA-protein complex, in which dsDNA appears to exist in an equilibrium between nicked and ligated states with relaxase (TraI) bound at the *nic* site (Zechner *et al.*, 1997; Byrd and Matson, 1997). However, transfer of the nicked strand does not occur until after the donor-recipient cell contact when the

nicked strand is unwound (Kinsman and Willetts, 1978). Unwinding of the nicked plasmid DNA is an activity of the relaxase/helicase TraI in the F plasmid (Matson *et al.*, 2001), which requires at least 20 nucleotides of single-stranded DNA upstream of the *nic* site (Csitkovits and Zechner, 2003). Although binding of relaxase at *oriT* also denatures the DNA around *nic*, the extent of the melted region does not appear to support TraI helicase activity (Zhang and Meyer, 1995; Guasch *et al.*, 2003). Therefore, a "mating signal" must be produced through donor-recipient cell contact, resulting in localized DNA melting at *oriT* to initiate DNA unwinding by TraI.

I propose that TraM responds to the unknown "mating signal" from the transferosome and induces localized melting between *nic* and *sbmABC* thereby allowing TraI to unwind DNA for conjugative transfer. Both TraM-*oriT* and TraM-TraD interactions are required for F conjugation in a way other than formation of the relaxosome or the transferosome (Chapter 5 and 6; Willetts and Wilkins, 1984), implying that TraM is essential for transferring the mating signal from the transferosome to the relaxosome during F conjugation. Although the F relaxosome normally resides at the cell center or quarter positions (Niki and Hiraga, 1997), formation of the donor- recipient mating pair might facilitate anchoring of the relaxosome at the transferosome through TraM-TraD interactions, with DNA unwinding following immediately. The quick conversion from dsDNA to ssDNA could be the reason that the R751 relaxosome was not observed to be coincident to the transferosome in mating donors using the LacO/LacI-GFP system, which recognizes only double-stranded DNA (Lawley *et al.*, 2002).

The "mating signal" could be a cascade of conformational changes that start from the formation of the donor-recipient mating pair. Gilmour *et al.* (2003) has shown that the

Figure 7-2. A model for TraM-TraD interactions in triggering DNA unwinding. (A) The *oriT* region bound by the relaxase domain of TraI ($TraI_R$), IHF, TraY (Y) and TraM tetramers (M4). DNA is in equilibrium between the nicked and ligated states. Both ends of the nicked strand attach to $TraI_R$. The localized melting caused by TraI binding at *oriT* is not enough for the helicase domain of TraI ($TraI_H$) to load. (B) After formation of the donor-recipient mating pair, *oriT*-bound TraM specifically interacts with the TraD and changes conformation, resulting in denaturation of the AT-rich region between *nic* and *sbmABC*. Consequently, $TraI_H$ enters upstream of *nic* and unwinds DNA for transfer.


(A)
M
M
M
nic
AT-rich
AT-rich
(B)
M
M
M

membrane associated R27TrhB (a transferosome component; F TraB homolog) interacts with TraG (the coupling protein; F TraD homolog). Conformational changes in the outer-membrane might be transferred to TraD via TraB, a TonB analog that interacts with the outer-membrane secretin-like TraK (Harris *et al*, 2001), resulting in an increased affinity of TraD for *oriT*-bound TraM, which might be different from the free TraM in conformation (Verdino *et al*., 1999). TraM-TraD interactions might further change the conformation of TraM at *oriT*, resulting in denaturation of the AT-rich region between *nic* and *sbmABC* (Figure 7-2A and B). The resulting single-stranded DNA upstream of *nic* allows the TraI helicase domain to load and unwind DNA from *nic* in a 5' to 3' direction (Lahue ad Matson, 1988; Csitkovits and Zechner, 2003). This hypothesis implicates TraD as an essential protein for DNA unwinding, agreeing with previous studies suggesting that TraD is also required for conjugative DNA synthesis (Kingsman and Willetts, 1978).

A single residue substitution at the C-terminal region of TraM appears to change the conformation of TraM thereby changing its DNA binding ability (Chapter 5). TraD interacts with multiple residues at the C-terminal region of TraM, which happen to overlap the tetramerization domain (Chapter 5 and 6), suggesting that TraM-TraD interactions might cause a conformational change in TraM. As TraM binds to three sites upstream of *nic* and probably forms a nucleosome-like structure similar to that proposed for RP4TraK (Di Laurenzio *et al.*, 1992; Ziegelin *et al*., 1992; Fekete and Frost, 2002), any conformational changes in TraM would have a greater potential to cause localized DNA denaturation. Of course, relaxosome-transferosome interactions might involve weak interactions between TraD and TraI or non-specific interactions between TraD and

plasmid DNA (Panicker and Minkley, 1992; Llosa *et al.*, 2003), which could also trigger denaturation of DNA at *oriT*. However, there has been no evidence that TraD-TraI interactions or TraD-DNA interactions are required for F conjugation.

It is impossible at this stage to provide a model to illustrate the signaling pathway during F conjugation, since many details, especially the requirement and extent of the localized DNA melting at *oriT*, need further investigation. The proposed mating signals, i.e. conformational changes in transfer proteins, also need to be proven. However, TraM does appear to be an essential signaling factor that transfers the mating signal from the mating pair to the relaxosome thereby triggering DNA transfer during F conjugation.

Chapter 8

Appendix: Analysis and Characterization of IncFV Plasmid pED208 Transfer Region

* A version of this chapter has been published: Lu, J., J. Manchak, W. Klimke, C. Davidson, N. Firth, R. A. Skurray, and L. S. Frost. 2002. Analysis and characterization of IncFV plasmid pED208 transfer region. Plasmid **48**:24-37.

8.1 Introduction

Plasmid pED208 (formerly EDP208) is a derepressed derivative of a naturally occurring *lac* plasmid, F$_0$*lac*, which was originally identified in *Salmonella typhi* (Falkow and Baron, 1962). Datta (1975) classified F$_0$*lac* plasmids within the IncF complex (IncFV; Finlay *et al.*, 1986b), which was supported by previous sequence analyses of some pED208 *tra* genes (Finlay *et al.*, 1986b; Finlay and Paranchych, 1986; Di Laurenzio *et al.*, 1991). pED208 is a multi-piliated derivative of F$_0$*lac* with approximately 17 pili per cell, which is 10-20 fold greater than other derepressed F-like plasmids (usually 1-2; Frost *et al.*,1985). The regions responsible for pilus synthesis and DNA transfer were previously mapped to two adjacent *Hin*dIII fragments on pED208, cloned into pBF101 and pBF105 (Finlay *et al.*, 1983). The *oriT* region, pilin gene *traA*, gene for the surface exclusion protein, TraT, and several other transfer genes were sequenced previously and were found to encode open reading frames (ORFs) homologous to transfer proteins encoded by other F-like plasmids. An IS*2* element was found inserted in the *traY* gene in pED208, which was regarded as the cause for multipiliation and transfer-derepression (Finlay *et al.*, 1986b). pBF101 is able to support the transfer of pBF105 suggesting that it encoded the entire region for pilus production as well as the coupling protein, TraD, (Cabezon *et al.*, 1997) and the relaxase, TraI, required for conjugative DNA transfer. The remainder of the *Hin*dIII fragment cloned in pBF101 was sequenced and analyzed and was compared to the F transfer region.

Table 8-1. pED208 transfer region sequence segments

Accession number	Coordinate (kb)[a]	Corresponding regions and genes	References
AF411480	-0.85-32.647	Entire pED208 *tra* region	This work
AY075109	(-0.85) - 0	gene *X*	This work
X59611	0 - 1.8	*oriT* , *traM*, *traJ*, and *traY* (partial)	Di Laurenzio *et al.*, 1991
V00279	1.8 - 3.1	IS2 sequence	Ghosal *et al.*, 1979
M14733	3.1 - 5.0	*traY* (partial), *traA*, *-L*, *-E*, *-K* (partial)	Finlay *et al.*, 1986b
AY046069	4.6 - 21.7	*traK*, *-B*,*-R*,*-V*,*-C*,*-W*,*-U*,*-N*,*-F*,*-H*, *-G*; *trbI*, *-C*, *-B*; and *orfX1*,*-2*,*-3*,*-4*	This work
M13465	20.5 - 24.0	*traS*, *traT* and *traD* (partial)	Finlay and Paranchych , 1986
AY043458	23.4 - 32.6	*traD*, *traI*, *orf4* and *traX*	This work

[a]The coordinates are the same as in Table 8-2.

8.2 Results and Discussion

8.2.1 Sequence of the pED208 transfer region

Using published sequences as a guide (Table 8-1), and pBF106, pBF111, pBF105 and pJLED1 as sequencing templates, the remaining 26-kb of the pED208 *tra* region on pBF101 and pBF105 was sequenced by primer walking (Table 8-1). The complete sequence of the pED208 *tra* region is available under the accession number AF411480. The *Eco*RI recognition sequence at the beginning of the *oriT* region defines coordinate 1. The first nucleotide upstream of the *Eco*RI site in the leading region defines coordinated -1. The 33.5kb sequence presented here represents positions 35.4kb to 7.5kb of the original *Hin*dIII/*Xho*I map of pED208 (Finlay *et al.*, 1983), which is about 3.9 kb longer than originally estimated. The pED208 *tra* region is 32.8kb in length, from the gene *X* to the end of *traX* (Figure 8-1). The overall G+C content of the pED208 *tra* region is 54.5% (F *tra* region is approx. 51.8%), while IncP plasmids have a much higher overall GC content (Thorsted *et al.*, 1998).

The pED208 *oriT* region contains a similar sequence (GGTGTGG; coordinates 54-60 on the lower strand) as the F *nic* site sequence, and thus was grouped into the F family (Frost *et al.*, 1994; Lanka and Wilkins, 1995). Immediately downstream of the putative *nic* site sequence, the pED208 *oriT* region contains an inverted repeat (GCAAGATTAAATCTTGC), which is similar to that in the F *oriT* region which has been implicated in termination (Di Laurenzio *et al.*, 1991; Gao *et al.*, 1994).

Analysis of the pED208 *tra* region further disclosed 35 putative open reading frames (ORFs), including four within the IS*2* element inserted in *traY*, unidirectionally aligned

Figure 8-1. Genetic organization of the pED208 *tra* region. The coordinates are the same as in Table 8-2. The clear boxes represent ORFs while the filled box stands for the IS*2* element inserted in *traY*. The triangle represents the *nic* site on the lower, transferred strand. Arrows indicate the location and the direction of promoters. Capital letters under the boxes stand for restriction sites: E for *Eco*RI; H for *Hin*dIII; B for *Bgl*II; X for *Xho*I.

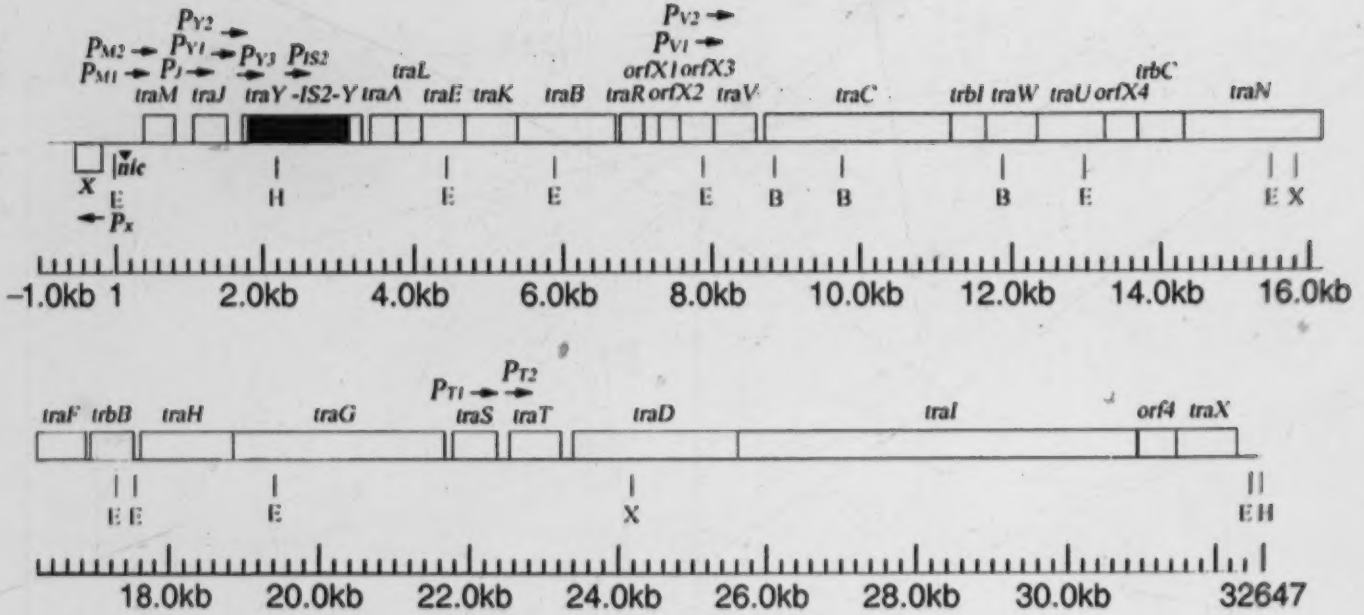

PY2
PM2
PY1
PY3
PIS2
PM1
PJ
traL
PV2
PV1
traM traJ traY -IS2-Y traA traE traK traB orfX1 orfX3 traR orfX2 traV traC trbI traW traU orfX4 trbC traN
nic
X
E
Px
H
E
E
E
B
B
B
E
E X
-1.0kb 1 2.0kb 4.0kb 6.0kb 8.0kb 10.0kb 12.0kb 14.0kb 16.0kb
PT1
PT2
traF trbB traH traG traS traT traD traI orf4 traX
E E
E
X
E H
18.0kb 20.0kb 22.0kb 24.0kb 26.0kb 28.0kb 30.0kb 32647

Table 8-2. Coding regions in pED208 transfer region[a]

Gene	*tra* region Coordinate	Aa number	pI	Aa identity[b]	Functions or predictions[c]
X	(-151) - (-537)	128		57	Transglycosylase
traM	393 - 776	127	5.7	37.8	*oriT* binding
traJ	1072 - 1521	149	7.6	12.8	Regulation of *tra* protein expression
traY	1727 - 1783; 3113 - 3295	79	NA	26.6	Disrupted by IS*2*; *oriT* binding and regulation
traA	3410 - 3769	119	10.1	33.6	Pilin subunit; LepI cleavage A55; T56 is acetylated
traL	3771 - 4076	101	9.8	46.2	Pilus assembly
traE	4096 - 4659	187	9.0	43.9	Pilus assembly
traK	4649 - 5389	246	9.8	35.1	Pilus assembly; possible hsp 70 chaperonin peptide-binding motif at 12-19; LepI cleavage after A21
traB	5376 - 6737	453	7.1	46.4	Pilus assembly
traR	6806 - 7072	88	7.6	37.0	Prokaryotic C4-type Zinc finger at 50-76
orfX1	7076 - 7261	61	9.6	NA	Type-A NTP binding site at 8-20
orfX2	7245 - 7535	96	9.8	NA	unknown
orfX3	7532 - 7978	148	7.6	NA	Chaperonin peptide-binding motif at 77-84
traV	7997 - 8611	204	10.0	33.9	Pilus assembly; LepII cleavage after G17; lipid modification at C 18
traC	8622 - 11213	863	6.3	53.8	Pilus assembly; Type-A NTP binding site at 478-489
trbI	11210 - 11674	154	8.8	42.2	RNA hairpin recognition motif at 32-41
TraW	11674 - 12309	211	8.5	44.8	Pilus assembly; FKBP-type peptidyl-prolyl *cis-trans* isomerase signature 1 at 144-161; LepI cleavage after A17
TraU	12306 - 13301	331	8.7	67.6	DNA transfer; LepI cleavage after A24
orfX4	13312 - 13680	122	9.3	NA	unknown
trbC	13677 - 14288	203	8.7	33.0	Pilus assembly
traN	14285 - 16138	617	6.1	46.7	Mating pair stability; possible LepI cleavage after L20
traF	16135 - 16914	259	6.1	40.9	Pilus assembly; possible LepI cleavage after A19

trbB	16936 - 17547	203	7.1	46.9	LepI cleavage site after A22
traH	17547 - 18908	453	8.3	49.0	Pilus assembly;LepI cleavage after A22
traG	18910 - 21744	944	8.6	33.2	Pilus assembly and mating pair stability
traS	21815 - 22375	186	9.2	13.4	Entry exclusion
traT	22552 - 23289	245	9.1	78.7	Surface exclusion; LepII cleavage after G22; lipid modification at C23
traD	23440 - 25650	736	6.4	48.2	DNA transport; Type-A NTP binding site at 187-199; Type-B NTP binding site at 422-428
traI	25650 - 30953	1767	6.1	44.5	*oriT* cleavage and unwinding; Type-A NTP binding site at 989-1001; Type-B NTP binding site at 1069-1075
orf4	30966 - 31520	182	11.2	45.6	unknown
traX	31535 - 32266	243	10.8	31.3	Pilin acetylation

[a] The ORFs of IS*2* (Table 8-1; Ghosal *et al.*, 1979) are not included here. pI (isoelectric point) and a.a. (amino acid) identities were determined by Peptool®.

[b] Aa identity refers to the percent amino acid identity between ORFs in the pED208 transfer region and corresponding proteins in the F transfer region (Frost *et al.*, 1994) except for ORF4 which is homologous to R100 ORF4 (Genbank accession No. NP_052987).

[c] Numbers refer to the Aa residue number in the ORF. Functions and processing are inferred from the homologous proteins encoded by the F *tra* region. Conserved motifs were detected by Peptool® or by referring to motifs in F *tra* proteins (Frost *et al.*, 1994). NTP-binding motifs were described by Walker *et al.* (1982) and Gorbalenya and Koonin (1990); the chaperonin binding epitope was described by Flynn *et al.* (1991); the lipoprotein modification site was described by Mattar *et al.* (1994); the prokaryotic C4-type Zinc finger was described by Doran *et al.* (1994); FKBP-type peptidyl-prolyl cis-trans isomerase signature was described by Wulfing *et al.* (1994); RNA hairpin recognition motif was described by Lazinski *et al.* (1989).

downstream of *oriT* except gene *X* (Figure 8-1), while the F *tra* region has 37 ORFs (Frost *et al.*, 1994). All the F *tra* genes essential for conjugation, except *traQ*, have homologues in the pED208 *tra* region and are in the same order except *traR* and *traV*. These genes are reversed in order and have three intervening ORFs (ORFX1, -X2, -X3; Figure 8-1; Table 8-2; Frost *et al.*, 1994). The gene products can be categorized into five groups based on functions inferred from their F homologues: pilin, pilus assembly and retraction (TraA, -L, -E, -K, -B, -V, -C, -W, -U, -F, -H, and N-terminal TraG; TrbC); mating pair stabilization (TraN and C-terminal TraG); surface and entry exclusion (TraT, -S); regulation and DNA processing (TraM, -J, -Y, -I); and DNA transport by the coupling protein (TraD; Cabezon *et al.*, 1997). The proteins in the first two groups are also named *Mpf* proteins since they are considered to be responsible for mating pair formation (*mpf*) between donor and recipient cells, an essential step for the conjugative transfer of DNA (Frost *et al.*, 1994).

Some genes (*finP, -O; traP, -Q; trbD, -G, -E,-A,-J,-F,-H; artA*) with known or unknown functions in the F *tra* region (Frost *et al.*, 1994) were not found in the *tra* region of pED208, while the pED208 *tra* region has four ORFs (ORF1,2,3,4) which have no obvious homology to other known proteins. ORF4 is highly homologous to R100 ORF4 which is not required for transfer (Genbank accession No. NC_002134). The leader peptidase cleavage site in F TraG (102 kDa) which is thought to be responsible for the periplasmic fragment, TraG* (50 kDa; Firth and Skurray, 1992), is not evident, suggesting that it might not be required for TraG function. The sequence information strongly supports the idea that the *tra* region of F_0*lac* and other F-like plasmids originated from the same archetype and evolved into closely related branches of the phylogenetic

tree as a result of the accumulation of random mutations and intermolecular and intercellular DNA transfer events.

8.2.2 Regulation of transfer gene expression in pED208

Putative promoters and transcriptional start sites in pED208 *tra* region were predicted and are shown in Figure 8-1. Two predicted *traM* promoters, PM1 and PM2, overlap the pED208 TraM binding sites determined by DNase I footprinting (Di Laurenzio *et al.*, 1991), suggesting that these promoters are autoregulated by TraM as is the case for the F plasmid (Penfold *et al.*, 1996).

Since *traY* is the first gene in the major *tra* operon, the *traY* promoter was thought to be the major promoter for the expression of transfer genes in F-like plasmids (Frost *et al.*, 1994). Three *traY* promoters (PY1, PY2, and PY3) were predicted in pED208 with two being located within the 3'-end of *traJ*, implying *in cis* regulation of PY by *traJ* as suggested for the R1 plasmid (Koronakis and Hogenauer, 1986). The insertion of an IS*2* element in *traY* introduced another putative promoter, PIS*2*. The introduction of this constitutive promoter could cause the overexpression of *tra* proteins, resulting in multipiliation of host cells (Finlay *et al.*, 1986b). However, if pED208 TraY is a negative autoregulator of the PY promoters, as in the case of R100 TraY (Taki *et al.*, 1998), the insertion of IS*2* could have inactivated *traY*, resulting in constitutive transcription from these promoters. Two promoters were predicted within *orfX3*, which could possibly aid in the expression of *traV* and following genes.

Two promoters were predicted to be present upstream of *traT*, with PT2 corresponding to that previously suggested, resulting in high levels of transcription of *traT* from its own promoter (Finlay and Paranchych, 1986).

An interesting feature of the pED208 transfer region is the lack of genes corresponding to *finP* or *finO* found in other F-like plasmids. These genes encode an antisense RNA system for regulating translation of *traJ* mRNA (Frost *et al.*, 1994; Jerome *et al.*, 1999). A derepressed mutant of F$_0$*lac*, F$_0$*lac*drd, has been isolated, suggesting that a fertility inhibition system is present on F$_0$*lac*, but the nature of this system has not been characterized (unpublished data). Although the predicted *traJ* transcript has a 124 base untranslated region similar to those in other F-like plasmids (Finlay *et al.*, 1986c), there are no inverted repeats characteristic of the stem-loop structures in FinP antisense RNA (Di Laurenzio *et al.*, 1991). Analysis of the sequence did not detect any predicted promoters that could initiate transcription of an RNA antisense to the *traJ* mRNA leader sequence.

ORF analysis of the sequenced region failed to reveal a FinO homologue (Table 8-2; Figure 8-1). FinO proteins from other F-like plasmids are almost identical (over 95% identity) and cross-reactive (Frost *et al.*, 1994). The *finO* genes occur either immediately after *traX* and *orf286* or after *traX* in Class I and II fertility inhibition systems, respectively (Cheah and Skurray, 1986; van Biesen and Frost, 1992). The sequence downstream of *traX* in pED208 contains a partial putative ORF starting at position 32308, which has no homology with any known *tra* proteins.

Since sequencing stopped at the *Hin*dIII site about 300-bp after *traX*, there existed the possibility that *finO* is within the unsequenced portion of pED208. To determine if

Table 8-3. Effect of F$_0$*lac* plasmid on transfer ability of derepressed F-like plasmids

Plasmids or plasmid combinations in donor cells	Transfer efficiency[a]		
	F$_0$*lac* or its derivatives	R100 or R100-1	pOX38-Km
F$_0$*lac*	6×10^{-4}	NA	NA
pED208	1×10^{-1}	NA	NA
F$_0$*lac*drd	1.5×10^{-1}	NA	NA
pOX38-Km	NA	NA	8×10^{-1}
R100	NA	1×10^{-3}	NA
R100-1	NA	1×10^{-1}	NA
F$_0$*lac* / pOX38-Km	8×10^{-3}	NA	2×10^{-1}
F$_0$*lac* / R100-1	2×10^{-2}	3×10^{-2}	NA
R100 / pOX38-Km	NA	1×10^{-3}	6×10^{-3}
F$_0$*lac*drd / R100	2×10^{-1}	1×10^{-4}	NA
F$_0$*lac*drd / pOX38-Km	2×10^{-2}	NA	8×10^{-3}
F0*lac*drd / R100-1	1×10^{-2}	5×10^{-3}	NA
R100-1/ pOX38-Km	NA	4×10^{-2}	2×10^{-1}

[a] The recipient strain ED24 carried non-transmissible pT75 to obtain ampicillin resistance. NA, not applicable.

there is a cross-reactive FinO protein encoded by F$_0$*lac* plasmids, mating experiments were carried out to determine the level of fertility inhibition of pOX38-Km and R100-1, which are derepressed due to the inactivation of their *finO* genes (Cheah and Skurray, 1986;Yoshioka *et al.*, 1987; Anthony *et al.*, 1999).

The repressed parental plasmid of pED208, F$_0$*lac*, transfers at 2-3 logs lower frequency than its transfer-derepressed derivatives, F$_0$*lac*drd or pED208 (Table 8-3). F$_0$*lac*, when co-resident with pOX38-Km or R100-1, did not cause a significant decrease (less than 10-fold) in their transfer efficiency; similarly, the transfer frequency of F$_0$*lac*drd, when co-resident with transfer- repressed R100, was not affected. As a positive control, the transfer-repressed plasmid R100 was found to decrease the transfer efficiency of pOX38-Km by 100-fold, which is consistent with earlier observations (Willetts and Maule, 1986). These results suggested that there is no cross-reactive FinO homologue encoded by F$_0$*lac* plasmids, which can complement the *finO* mutations in F or R100-1, and *vice versa*, FinO protein expressed by R100 cannot repress the transfer of derepressed F$_0$*lac* plasmids.

8.2.3 Plasmid-specific features of conjugation-related proteins encoded by the pED208 *tra* region

Although the *tra* region of pED208 is very homologous with the *tra* regions of other F-like plasmids, it still has many plasmid-specific characteristics. Besides the above-mentioned differences in regulation, specificity has also been found for pED208 pili synthesis and surface exclusion. The pili expressed by F and pED208 are serologically distinct (Bradley and Meynell, 1978), although pED208 pili are still sensitive to the F-

specific filamentous DNA phages (Bradley, 1981). In pED208, there is no ORF homologous to F *traQ*, which encodes a chaperonin important for F pilin maturation and F transfer (Moore *et al.*, 1982; Frost *et al.*, 1994). However, several ORFs in pED208 contain hsp70 chaperonin peptide-binding motifs (Table 8-2), which might substitute for F TraQ. Alternatively, there could be no requirement for a pilin chaperone in F$_0$*lac* plasmids, which might contribute to the multipiliated phenotype of pED208-containing cells.

F and R100 could be transferred into host cells containing F$_0$*lac* and pED208, and *vice versa* (data not shown), indicating that the surface exclusion system for F$_0$*lac* plasmids is plasmid-specific. Interestingly, the TraT proteins of pED208 and F share the highest homology among all the *tra* proteins of the two systems, while TraS sequences among F-like plasmids share exceptionally low homology (Table 8-2; Finlay and Paranchych, 1986; Frost *et al.*, 1994). However, high homology does not necessarily preclude plasmid specificity, since a single amino- acid difference between F and R100 TraT defines the specificity for surface exclusion in these two systems (Harrison *et al.*, 1992; Frost *et al.*, 1994). The sequences determining the specificity of pED208 TraT has not yet been reported although sequence differences in the TraT of F, R100, and F$_0$*lac* have been discussed (Sukupolvi and O'Connor, 1990).

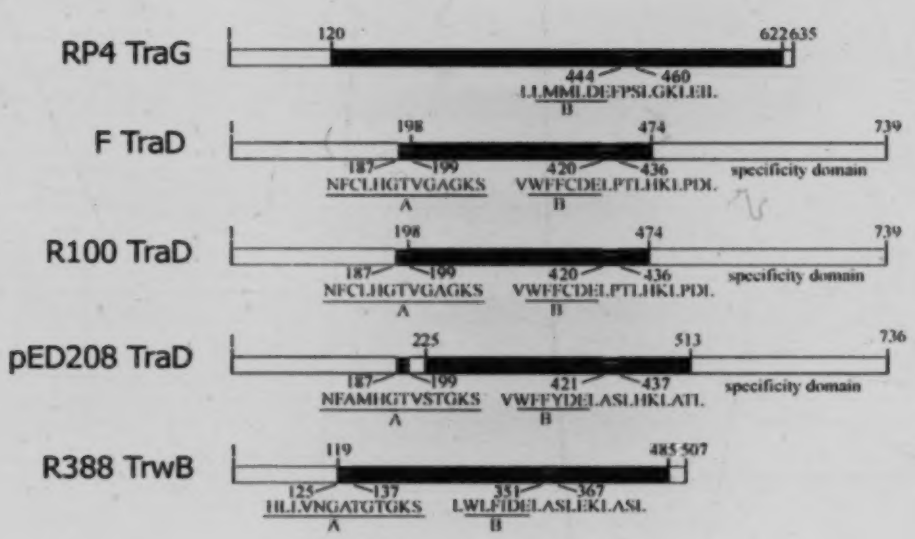
As determined by mobilization assays, F, R100 and pED208 showed specificity for their own *oriT* regions (Table 8-4). Plasmids containing *oriT* and the complete *traM* operon, encoding the essential *tra* protein, TraM, which is highly plasmid-specific (Kupelwieser *et al.*, 1998; Fekete and Frost, 2000), were used in mobilization assays. These plasmids, pLDL100, pNY300 and pRF105, which were derived from the *oriT*

regions of pED208, F and R100, respectively (Table 2-1), could only be mobilized by their cognate parental plasmid. This suggests that there are additional layers of specificity beside that defined by TraM in defining the mobilization potential of a plasmid.

This additional specificity could be defined by the binding of TraY to *oriT*, the specific interaction between the relaxase, TraI, and *oriT* to form the relaxosome (TraI,-M,-Y), or the specific interactions between the transferosome (TraD and *mpf* proteins) and the relaxosome. TraY has been found to be required for the formation of relaxosomes and the efficient mobilization of *oriT* constructs in F and R100 transfer (Nelson *et al.*, 1995; Fekete and Frost, 2000). However, pOX38-*traY*244 can still transfer at low but detectable frequencies (Maneewannakul *et al.*, 1996; Penfold *et al.*, 1996), while R1 TraY is not essential for nicking and mobilization of R1 *oriT* constructs (Kupelwieser *et al.*, 1998; Karl *et al.*, 2001). The *traY* gene in the pED208 *tra* region is interrupted by IS*2* (Figure 8-1; Table 8-2; Finlay *et al.*, 1986b), suggesting that either the insertion did not destroy TraY function or that F_0*lac* TraY is not required for transfer. Interestingly, F_0*lac* (pED208) and R1 share the same pattern of short repeats within their *oriT* regions, suggesting that they are evolutionarily related in terms of relaxosome construction (Frost *et al.*, 1994).

Previous observations suggested that F *mpf* mutants, but not mutants in DNA metabolism genes, could utilize the pED208 transfer apparatus to support transfer at lowered frequencies (Finlay *et al.*, 1986b). Interestingly, although F_0*lac* could not repress the F-like plasmids, pOX38-Km or R100-1, its transfer efficiency was enhanced by o1-2 logs when it coexisted with pOX38-Km or R100-1 (Table 8-3). However, the transfer efficiency of R100 did not increase when coexistent with F_0*lac*drd. Perhaps F_0*lac* can

Figure 8-2. Schematic diagram of the RP4 TraG, F, R100 and pED208 TraD, and R388 TrwB. Numbers indicate amino acid residues from the N-terminus. TraG domains were detected by BLAST search and are shown as filled boxes. Conserved motifs are shown as lined boxes. Specificity domains of TraD proteins have not been precisely mapped and are shown here to indicate the C-termini of TraD proteins. Type-A and Type-B NTP binding sites are underlined and noted as A and B, respectively.


RP4 TraG
120
622635
444
460
LLMMLDEFPSLGKLEIL
B
F TraD
198
474
739
187
199
420
436
specificity domain
NFCLHGTVGAGKS
VWFFCDELPTLHKLPDL
A
B
R100 TraD
198
474
739
187
199
420
436
specificity domain
NFCLHGTVGAGKS
VWFFCDELPTLHKLPDL
A
B
pED208 TraD
225
513
736
187
199
421
437
specificity domain
NFAMHGTVSTGKS
VWFFYDELASLHKLATL
A
B
R388 TrwB
119
485-507
125
137
351
367
HLLVNGATGTGKS
LWLFIDELASLEKLASL
A
B

utilize F and R100-1 transferosomes to augment its transfer efficiency while R100 cannot use the F$_0$*lac*drd system. Since the transfer frequency of F$_0$*lac* in the presence of pOX38-Km or R100-1 did not reach that of pED208, the ability to borrow the transfer apparatus of other plasmids appears to be limited. This could be due to a requirement for the derepressed expression of a protein specific for F$_0$*lac* systems.

When transfer-derepressed F$_0$*lac*drd coexists with other F-like derepressed plasmids such as pOX38-Km or R100-1, each plasmid transfers at a lower frequency, exhibiting a significant level of mutual transfer repression (Table 8-3). pOX38-Km and R100-1 co-residing in the same cell did not show a comparable phenotype. These results suggested that compatibility between the F$_0$*lac* transfer system and F (or R100) transfer system exists but is limited in some way. When both systems are expressed at derepressed levels, they appear to interact with each other, leading to a mutual negative dominant effect for conjugative DNA transfer. One possible explanation is that there are limited sites within the cell for construction of the transferosome and F$_0$*lac* plasmids compete with other derepressed F-like plasmids for these sites. pED208 was unstable when coexistent with pOX38-Km (data not shown), suggesting that competition for these pilus assembly sites might extend to competition for sites for replication or partitioning in a highly overexpressed system.

8.2.4 Specificity for the coupling protein, TraD

The amino acid sequence of the pED208 *traD* gene product shares high homology with the sequences of F and R100 TraD, and R388 TrwB (Jalajakumari and Manning, 1989; Yoshioka *et al.*, 1990; Llosa *et al.*, 1994), which are members of the TraG family

Table 8-4. Transfer efficiency of mobilizable plasmids, or of self-transmissible plasmids

Mobilizable plasmids in donor cells with self-transmissible plasmids	Transfer efficiency			
	Donors with pED208		Donors with pOX38 derivatives[a]	
	Mobilization	Self-transmission	Mobilization	Self-transmission
R1162	5×10^{-7}	1.2×10^{-1}	4×10^{-7}	3×10^{-1}
pCR1	1.5×10^{-1}	1×10^{-1}	5×10^{-1}	2×10^{-1}
pLDL100	2	8×10^{-2}	$< 1 \times 10^{-7}$	2×10^{-1}
pNY300	3×10^{-5}	1×10^{-1}	8×10^{-1}	1×10^{-1}
pRF105	1×10^{-5}	8×10^{-2}	5×10^{-6}	1×10^{-1}

[a] As pCR1 is Km resistant, pOX38-Tc was used to mobilized pCR1 instead of pOX38-Km.

of proteins (Lessl *et al.*, 1992). pED208 TraD contains the Type-A NTP binding site also found in these homologues (Figure 8-2; Cabezón, *et al.*, 1997), while RP4 TraG and some other TraG family proteins do not have this motif (Lessl *et al.*, 1992). This motif could be functionally important for pED208 transfer since a mutation (K136T) in this motif in TrwB, a homologue in the IncW R388 plasmid system, is completely deficient for plasmid transfer (Moncalian *et al.*, 1999). pED208 TraD also contains a sequence conserved amongst the entire TraG family of proteins named "motif B" in RP4 TraG (Balzer *et al.*, 1994). This sequence consists of a Type-B NTP-binding site (Figure 8-2), which could be essential for TraG family function, based on the transfer-deficient phenotype of a D449N mutation in RP4 TraG (Balzer *et al.*, 1994). The importance of these two motifs was further substantiated by the recent finding that the nucleotide-binding site seen in the crystal structure of TrwB is defined by two segments containing the Type-A and Type-B NTP binding motifs (Gomis-Ruth *et al.*, 2001).

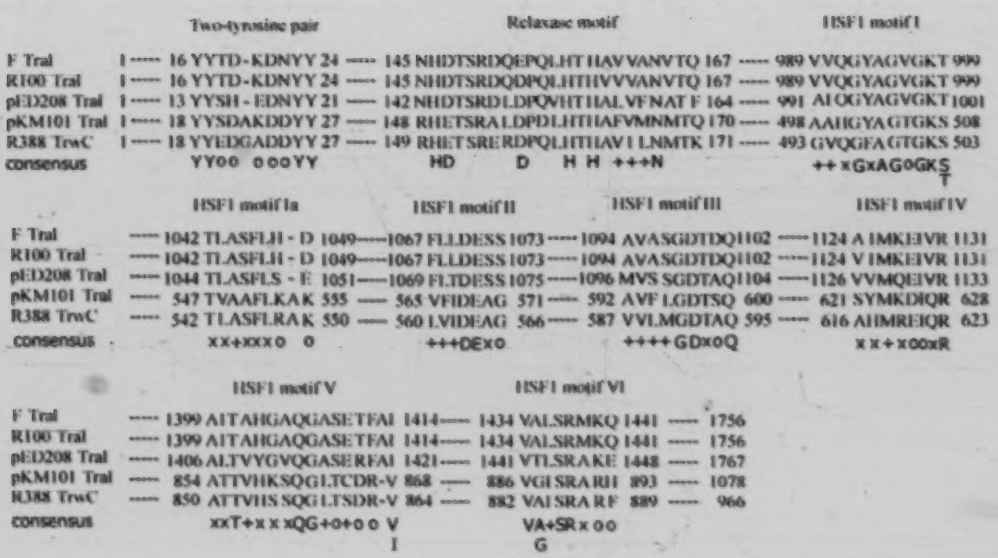
Mobilizable plasmids ColE1 and R1162 (an IncQ plasmid almost identical to RSF1010) encode their own DNA processing proteins for relaxosome formation but require self-transmissible plasmids, such as F or RP4, to provide a compatible transfer apparatus for efficient mobilization (Warren *et al.*, 1979; Meyer *et al.*, 1982). While both F and RP4 mobilize ColE1, only RP4 can efficiently mobilize RSF1010 or R1162. Recently, a C-terminal deletion in F TraD resulted in efficient transfer of R388 or RSF1010 (Sastre *et al.*, 1998) suggesting that the extension at the C-terminus of F-like TraD proteins, which is not present in RP4 TraG-like proteins, blocks mobilization of these plasmids. Based on this observation, pED208 would be predicted to mobilize IncQ plasmids poorly since it resembles F TraD more than RP4 TraG (Figure 8-2).

Mobilization assays of the ColE1 derivative, pCR1, and R1162 were carried out in the presence of pED208 or pOX38-Km (or pOX38-Tc; Table 8-4). pED208 was found to mobilize pCR1 very efficiently and R1162 poorly, which is similar to the pOX38-based plasmids. These results suggested that the extended C-terminal domain in pED208 TraD prevented efficient R1162 transfer, however, deletion analysis would have to be done to confirm this result. The C-terminal domain could serve to determine the specificity of interaction with the relaxosomes of its own and closely related plasmids (such as ColE1), and prevent the interaction with other relaxosomes of comparatively remotely related plasmids (such as IncQ plasmids). Based on the differences in conserved sequences and the mobilization specificity, TraD proteins of F-like plasmids and TraG proteins of IncP plasmids can be regarded as two different groups within the TraG family, which are evolutionarily adapted to their own transfer systems (Sastre *et al.*, 1998).

8.2.5 Relaxase of the pED208 transfer system

The amino acid sequence translated from pED208 *traI* contains a relaxase motif common to all the relaxases in different transfer systems (Pansegrau and Lanka, 1991; Figure 8-3) with pED208 TraI sharing the highest homology with the relaxases of IncFI (F), IncFII (R100), IncW (R388) and IncN (pKM101) transfer systems. These proteins contain a conserved N-terminal two-tyrosine pair motif, which has been suggested to be important for the nicking and religation of DNA during F and R388 conjugation (Frost *et al.*, 1994; Byrd and Matson, 1997; Grandoso *et al.*, 2000). RP4 TraI and some other relaxases in related DNA transfer systems share much lower homology with these relaxases. Only a single tyrosine (Y22) is present at the N-terminus of RP4 TraI,

Figure 8-3. Conserved motifs in the sequences of the F-family relaxases. Numbers indicate the sequence positions of the first and last amino acid residues of the motifs. F TraI sequence (Bradshaw *et al.*, 1990); R100 TraI sequence (Yoshioka *et al.*, 1990); pKM101 TraI sequence (Genbank accession No. AAB97287); R388 TrwC sequence (Llosa *et al.*, 1994). The consensus for the relaxase motif and HSF1 (helicase superfamily 1) motifs refer to that specified by Pansegrau and Lanka (1991) and Hall and Matson (1999), respectively. '+' represents a hydrophobic residue; 'o' represents a hydrophilic residue and 'x' represents a residue that is not restricted to being hydrophobic or hydrophilic.


Two-tyrosine pair
Relaxase motif
HSF1 motif I
F Trai 1 ----- 16 YYTD-KDNYY 24 ----- 145 NHDTSRDQEPQLHTHAVVANVTQ 167 ----- 989 VVQGYAGVGKT 999
R100 Trai 1 ----- 16 YYTD-KDNYY 24 ----- 145 NHDTSRDQDPQLHTHVVVANVTQ 167 ----- 989 VVQGYAGVGKT 999
pED208 Trai 1 ----- 13 YYSH-EDNYY 21 ----- 142 NHDTSRDLDPQVHTTIALVFNATF 164 ----- 991 AIQGYAGVGKT 1001
pKM101 Trai 1 ----- 18 YYSDAKDDYY 27 ----- 148 RHETSRALDPDLHTHAFVMNMTQ 170 ----- 498 AAHGYAGTGKS 508
R388 TrwC 1 ----- 18 YYEDGADDYY 27 ----- 149 RHETSRERDPQLHTHAVILNMTK 171 ----- 493 GVQGFAGTGKS 503
consensus YY00 000YY HD D H H +++N ++xGxAG0GKS T
HSF1 motif Ia
HSF1 motif II
HSF1 motif III
HSF1 motif IV
F Trai ----- 1042 TLASFLH - D 1049 ----- 1067 FLLDESS 1073 ----- 1094 AVASGDTDQ 1102 ----- 1124 A IMKEIVR 1131
R100 Trai ----- 1042 TLASFLH - D 1049 ----- 1067 FLLDESS 1073 ----- 1094 AVASGDTDQ 1102 ----- 1124 V IMKEIVR 1131
pED208 Trai ----- 1044 TLASFLS - E 1051 ----- 1069 FLTDESS 1075 ----- 1096 MVS SGDTAQ 1104 ----- 1126 VVMQEIVR 1133
pKM101 Trai ----- 547 TVAAFLKAK 555 ----- 565 VFIDEAG 571 ----- 592 AVF LGDTSQ 600 ----- 621 SYMKDIQR 628
R388 TrwC ----- 542 TLASFLRAK 550 ----- 560 LVIDEAG 566 ----- 587 VVLMGDTAQ 595 ----- 616 AHMREIQR 623
consensus xx+xxx0 0 +++DExo ++++GDx0Q x x + x00xR
HSF1 motif V
HSF1 motif VI
F Trai ----- 1399 AITAHGAQGASETFAI 1414 ----- 1434 VALSRMKQ 1441 ----- 1756
R100 Trai ----- 1399 AITAHGAQGASETFAI 1414 ----- 1434 VALSRMKQ 1441 ----- 1756
pED208 Trai ----- 1406 ALTVYGVQGASERFAI 1421 ----- 1441 VTLSRAKE 1448 ----- 1767
pKM101 Trai ----- 854 ATTVHKSQGLTCDR-V 868 ----- 886 VGISRARH 893 ----- 1078
R388 TrwC ----- 850 ATTVHSSQGLTSDR-V 864 ----- 882 VAISRARF 889 ----- 966
consensus xxT+xxxQG+0+00V I VA+SRx00 G

which has been suggested to be essential for nicking and religation of DNA during conjugation (Balzer *et al.*, 1994; Frost *et al.*, 1994). Thus, like TraD, pED208 TraI could be grouped into a relaxase family represented by F, which is distinct from the relaxase family represented by RP4 TraI, as previously suggested (Ilyina and Koonin, 1992; Byrd and Matson, 1997).

pED208 TraI was found to contain a C-terminal helicase domain also found in the F-family relaxases. F and R100 TraI and R388 TrwC contain both relaxase and helicase activities with the helicase domains belonging to helicase superfamily 1(HSF1) and containing all seven of the conserved motifs (Figure 8-3). The importance of helicase activity being physically linked to relaxases has been discussed previously for the R388 and F transfer systems (Llosa *et al.*, 1996; Matson *et al.*, 2001).

8.3 Conclusions

pED208 and its parental plasmid F$_0$*lac* clearly belong to the F family of type IV secretion systems (Christie and Vogel, 2000). As evidence accumulates about the similarities and differences among the members of this group, it is clear that two subfamilies can be defined based on a few salient characteristics. All of the Type IV systems have a pilus-based transport mechanism for protein, nucleoprotein complex or nucleic acid with the ability to transport nucleic acid dependent on the presence of a TraG (or TraD) homologue. The systems that resemble TraG in RP4 or the *vir* genes in the Ti plasmid appear to be able to transport nucleoprotein complexes. They have a TrbB/VirB11 homologue and lack a helicase function as well as the mating pair stabilization proteins, TraG and TraN. The F-like type IV systems appear to transport naked DNA into the recipient cell. They lack a TrbB function but have a helicase either as a domain within the relaxase or as a separate protein. They also have TraG and TraN homologues. Examples of F-like systems are fewer than for RP4 and Vir systems but the list is growing. Aside from F-like conjugative plasmids found in enteric bacteria, R27 of the IncH complex (Sherburne *et al.*, 2000), pNL1 in *Sphingomonas aromaticivorans* (Romine *et al.*, 1999) and a chromosomally encoded mechanism for transporting DNA into the medium in *Neisseria gonorrhoea* (Dillard and Seifert, 2001), are found in this group. F$_0$*lac* is an interesting example of the F subgroup since it is similar to F in many ways yet there has been considerable sequence divergence to the point where homology at the DNA level is undetectable by southern blot (Finlay *et al.*, 1986b). Coupled with the ability of pED208 to stably express pili at a very high level, it might prove to be a useful tool for studying conjugative pilus structure and function.

Chapter 9

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